DEVELOPMENTAL GENETICS OF SPOTTING PATTERNS IN THE MOUSE

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INTRODUCTION

Piebald spotting has usually been treated as a quantitative characteristic in genetic analyses of the past. The amount of pigment has often been measured for the coat as a whole, but little attention has been given to the developmental processes through which genes act to make one region differ from another. In this study variegated mice have been used to determine the effects of mutant genes on the many pigment cell lines which contribute to the whole. The pigment cell lines in different areas of the variegated coat exhibit different phenotypes; presumed changes in gene action serve to identify one line from another.

The life processes of pigment cells must be given careful consideration since their activities are responsible for the color patterns. Evidence that the processes of differentiation, mitosis and death of pigment cells are under genetic control has been found in comparative studies of normal, mutant and variegated animals. The variegated types have been particularly instructive for they often show the activities of both wild-type (normal) and genetically altered cells within the same individual. Similar genetic changes are believed to operate in the development of diverse cell types in neoplasms (Schultz, 1959) and aging tissues (Strehler, 1960; Gowen and Gowen, 1961). Thus, analyses of pigmentation patterns, which are relatively easy to observe, may supply basic information which is difficult to obtain in studies of other cell types.

Pigment cells have many attributes which render them excellent material for studies of general cell biology. They can be made to

proliferate, migrate, and differentiate in tissue culture as well as in the living organism. By mutation or experimental treatment their activities in vivo can be extended or restricted within relatively wide limits without endangering the life of the harboring organism. Pigment cells are very sensitive to many teratogenic agents; a change in their activity is a valuable indicator in the bio-assay of irradiation (Chase, 1958; Quevedo and Grahn, 1958) or chemical treatment (Landauer, 1954). Genes which primarily affect pigment cells have proven very useful in determining the frequencies of radiation induced genetic changes at specific loci in both the germ cells (Russell and Russell, 1959), and somatic cells (Russell and Major, 1957). The patterns formed by the deposition of melanin in the hair or feather are a permanent record of the physiological activities of the pigment cells as attested by Gluecksohn-Waelsch (1951, p. 7)

> Since hair is being shed and replaced frequently during the lifetime of the mouse, the developmental events leading to pigment formation repeat themselves and can thus be observed continuously within the same organism. The hair, with its differences in pigmentation along the hair shaft, is an image of the processes going on in the hair bulb during the formation of pigment as determined by the individual genes of the particular cell.

and by Lillie (1942, p. 259)

> The finished feather records permanently in its colour, pattern and structure the results of fluctuations of condition whether produced by experiments or otherwise. There is no more convenient or accurate record than the feather once one has learned how to identify the locus of reaction in the germ with the result in the finished feather; in short, to read its autobiography.

A study of pleiotropic genes, which produce anomalies of pigmentation among their effects, also reveals that the biology of cells in

general is probably very similar to that of the pigment cell. There is evidence that the metabolic activity of melanoblasts, germ cells, and blood cells is disturbed in a similar way in all three cell types in the W mutants of the mouse (Mintz, 1960). Sterility, anemia, deafness, choreic behavior, anomalies of the axial skeleton and even lethality are frequently associated with coat color dilution and/or piebald spotting. Grüneberg (1952, pp. 75, 248) commented that the white regions of the piebald spotting patterns often corresponded to embryonic regions in which development was retarded. Regardless of whether the retardation had transitory or permanent effects on other tissues, the spotting pattern remained as a permanent record of embryonic disturbances.

Of the many colors and patterns which can be observed in mammals and birds, two particular mutant types were investigated in this study. In one type, which will be referred to as "piebald spotting", pigmented spots occur on a white background. Familiar examples are Holstein-Friesian cattle, spotted cats, and spotted guinea pigs. Allen (1914) concluded that the pigment spread out from centers on a non-pigmented background in piebald spotted types. As an analogy, the spreading out of ink spots on a white sheet of paper results in a similar pattern of separate and contiguous spots. It follows that the pigmented areas, rather than the white regions, should be considered as the "spots" even if most of the coat becomes pigmented by the spreading process. The point may seem trivial, but failure to define "spot" has rendered the literature on spotting ambiguous on many occasions. In the second type of spotting, which will be called "variegation", two or more distinct phenotypes

involving pigment occur on the same animal. One phenotype may form the background color on which flecks or small spots of another type appear. The small spots seem to develop by the spreading of pigment from sites at which qualitative changes in pigmentation take place. When the areas of contrasting phenotypes approach equality in size, they usually develop from separate centers; each area expressing a different phenotype may then be considered as a spot. Familiar examples of variegation spotting are Blue Andalusian chickens, tortoiseshell cats, and tortoiseshell guinea pigs.

The reference to contrasting phenotypes of pigmentation implies that variegation spotting types are genetic mosaics. There is evidence that the pigment cells of contrastingly colored spots differ in genotype. Genetic mosaics should not be confused with patterns of contrasting colors which occur regularly in animals as a result of different physiological effects within the same genotype. For example, wild-type barring of feathers in birds (Rawles, 1960) and banding of hair in agouti mammals (Silvers, 1961) are the result of melanoblasts differentiating into two kinds of melanocytes, which produce eumelanin and pheomelanin, even though the cells do not change in genotype. Barring and agouti patterns are relatively consistent from animal to animal. In variegation spotting types it is not possible to predict which pigment areas will be colored differently; alterations in color presumably are dependent on chance genetic changes rather than solely on physiological processes. Bhat (1949) described a mosaic mouse which was a combination of both genetic and physiological patterns. It was a mosaic type by virtue of showing areas

of contrasting genotypes, white-bellied agouti ($\underline{A}^{W}$) and non-agouti ($\underline{a}$), but the black and yellow banding of the hairs within the agouti areas and the contrast between the agouti dorsum and yellow ventrum were physiological in nature.

Because so many cases of mosaicism had occurred in piebald spotted animals, Dunn (1934) suggested that somatic mitosis was frequently irregular in piebald spotting mutants. Schaible (1959) and Schaible and Gowen (1960) found that the frequency of mosaicism involving $\underline{Mi}^{wh}$ heterozygotes of the mouse was actually lowered when the homozygous state of piebald ($\underline{s}$) was included in the genotype. The effects of piebald spotting on the frequencies of mosaicism is given further consideration in this thesis.

Because so much information on the embryogenesis of pigment cells in the chick is available, an experiment on the rate of pigment spread in the chick was included in this study to compare the development of piebald spotting patterns in the mouse and chicken. No typical piebald spotting patterns can be found in adult chickens (Hollander, 1944). Temporary piebald spotting patterns do occur in chicks of certain mutant combinations (Punnett and Pease, 1927) or chicks developed from embryos treated with teratogenic compounds (Landauer, 1954).

The objectives of this investigation are:

1. To determine whether or not piebald spotting and variegation spotting can be considered as developmentally distinct types of pigmentation patterns in mammals and birds.
2. To test the hypothesis that the pigmented spots of piebald and

variegation spotting patterns mark the extent of migration of the melanoblasts.

3. To review the mechanisms of genetic mosaicism in regard to their possible application to the various types of mosaicism found in mammals and birds.
4. To determine the most likely mechanisms of mosaicism in $\underline{Mi}^{wh}$, $\underline{W}^{a}$ and $\underline{Va}$ mutants of the mouse.
5. To determine whether or not genetic change takes place in the germ cells as well as the pigment cells in $\underline{Mi}^{wh}$, $\underline{W}^{a}$ and $\underline{Va}$ heterozygotes.
6. To determine the effects of piebald spotting genes and *in utero* irradiation on the frequency of $\underline{Mi}^{wh}/+$ mosaic individuals and on the frequency of genetic change within specific areas of their coats.

The major heading which follows is entitled "Discussion of Literature" rather than the conventional "Review of Literature" because it seems necessary to show how previous work can be integrated to form the base upon which the present study rests. Discussion at this point allows for the presentation of hypotheses based primarily on a review of literature and thus eliminates repetition of many references to literature in the "Discussion of Results". Although the writer's own experience undoubtedly is reflected in the presentation of the literature, his discussion allows the reader to interpret the literature and approach the problem with the same viewpoint. This method of presentation seems justified, since the reader always has the option of examining the

literature himself and interpreting the material in light of his own experience.

Though the mouse and, to a much lesser extent, the chicken were used as the experimental animals in this study, it is necessary to consider what information is available on piebald and variegation spotting in other species of mammals and birds. Any hypothesis proposed to explain the development of spotting patterns must eventually stand the test of application to other species since the patterns are so widespread. Objections to the hypothesis of retarded melanoblast migration seem to stem from a lack of definition and classification of developmental types rather than any actual conflicting data. An attempt is made to eliminate some of the confusion in the discussion of literature. Because the mechanisms of mosaicism have been worked out much more thoroughly in some species of insects and plants, it is necessary to examine the literature on those forms in order to gain leads for experimental techniques and interpretation of mosaicism in mammals and birds.

In the first major subheading under "Discussion of Literature", the developmental means by which mutant genes cause whiteness in the coats of mammals and birds is reviewed. Evidence is presented to show that there is a developmental basis for distinguishing piebald spotting mutants from other mutants exhibiting white regions in their coats. Essentially, migration of melanoblasts is restricted in piebald spotting mutants so that certain regions remain void of pigment cells and consequently are white in appearance. In other mutants the pigment cells are distributed over the entire body, but for some reason they produce no pigment in

localized areas or none at all as in some pure white types.

Not all animals which exhibit more than one color of pigment in their coats are variegation mosaics. Of those which qualify as variegation mosaics, the genetic change is not always in the pigment cells. But when the change does take place in the pigment cells, the characteristic pattern of a mosaic variegation mutant develops. In some of the other mutants, including some variegated ones, the control of pigment production is not autonomous with the pigment cells but rather is dependent on the tissue environment. In the third major subheading the developmental characteristics of the various multiple color types are used to distinguish variegation spotting as a separate type of pattern.

Because the number of references on spotting patterns and mosaicism is so great, both the original references and recent reviews cannot be cited in all cases. If, in the writer's opinion, the pertinent material was covered adequately and the original reference was cited in the review, the earlier reference will usually be omitted.

Nomenclature of pigment cells in this thesis will follow that adopted by the Third Conference on the Biology of Normal and Atypical Pigment Cell Growth (Gordon, 1953).

Melanoblast: an embryonic cell potentially capable of producing melanin.

Melanocyte: a mature melanin-producing and melanin-containing cell.

Wild-type will be used as a standard of reference in describing the effects of mutations and in genetic notation (Hollander, 1953). Only the mutant genes affecting the characteristic under consideration will be designated. All other genes known to affect the characteristic can be

assumed to be wild-type. This notation, developed by geneticists working with Drosophila, has essentially been accepted by The Committee on Standardized Genetic Nomenclature for Mice (Dunn et al., 1940; Snell et al., 1960) and its use has been proposed for poultry (Jaap and Hollander, 1954).

DISCUSSION OF LITERATURE

Factors Determining the Absence of Pigment

Piebald spotting mutants

Allen (1914) formulated an hypothesis to account for the piebald spotting patterns of mammals and birds. Because the pigmented spots tended to be round in shape and consistent in location, Allen concluded that pigment spread out from eleven centers on the body: a medial coronal center and 5 bilaterally paired centers; aural, nuchal, scapular, pleural and sacral. It appeared that normally the spreading was extensive enough to pigment the entire coat. But in piebald spotting types the amount of pigment appeared to be limited; restriction of the spreading resulted in white gaps between the pigmented areas. Allen referred to the non-pigmented gaps as primary breaks; any breaks within the pigment areas he called secondary breaks.

Allen did not suggest the means by which pigment spread out from the centers. However, Goodale and Morgan (1913) had already proposed a theory to account for the pigmented spots on the piebald spotted guinea pig. They suggested that each pigmented spot contained a separate population of cells proliferated from a given cell which produced a color effect quite different from the surrounding white region.

Wright (1917) described, in addition to piebald spotting, the tortoiseshell type and the combination of the two patterns in the guinea pig. The tortoiseshell guinea pig shows no white; the pattern is made up of wild-type regions and yellow regions arranged in irregular alternate

bands across the back. In the combination type, it was apparent that the piebald spotting and tortoiseshell patterns were in some way related in development rather than just superimposed. The transverse wild-type stripes and yellow stripes were reorganized into wild-type spots and yellow spots against a non-pigmented background. When both wild-type and yellow colors were present in the same spot, the center tended to be wild-type and the periphery yellow. Wright concluded that either there was a radiation of some influence from centers in the skin or else mutation in the somatic cells was giving rise to different colored spots. After the discovery in the 1930's that the pigment cells migrated to the epidermis from the neural crest in amphibians and birds (Ris, 1941), Chase (1939) and Wright (1942) suggested that arrested migration of pigment cells in spotted mammals resulted in pigmented spots which were restricted in size.

Rawles (1947) demonstrated by transplantation experiments with the C57 strain of mice that the pigment cells of mammals also migrated out from the neural crest. Passage of the melanoblasts through specific regions between the neural crest and the limb buds was detected by transplanting tissues from specific regions to chick coelom. If the tissue was excised before the melanoblasts had passed through the region, white hairs developed in the graft. If melanoblasts were present in the region, they were revealed when they became differentiated in the graft. Initiation of migration took place in the anterior levels of the 8-day embryo and progressively later in the posterior regions as the embryo developed. Grafts of the dorsal ectoderm and underlying mesoderm of the

proximal part of the limb buds were the last examined regions to show arrival of melanoblasts; sites more distal on the legs were not investigated. Thus, if migration could be arrested by a mutation or treatment, the feet, tail tip, and mid-ventrum would be among the most likely regions to remain white.

Grüneberg (1952, p. 75) reviewed studies which showed that the tail tip and mid-ventral regions are characteristically white in the C57BL strain. By appropriate crosses between the C57 strain and the CBA strain, which has a normally pigmented tail, Grüneberg demonstrated that a single mutant gene was responsible for the segregation of the white tail tip. Pigmentation of the feet and belly were not classified. Silvers (1956) could not detect any melanocytes in the hair follicles of the white regions of the C57BR/cd mice or 7 other piebald spotting mutants of the mouse. The melanocyte complement of the pigmented areas was normal.

Grüneberg (1952, pp. 75, 248) in reviewing the piebald spotting effects of mutants in the mouse commented that the white markings frequently corresponded to regions of transitory embryonic disturbances. In addition to piebald spotting, the pleiotropic effects of such mutants may include dilution of color and anomalies of the axial skeleton, nervous system, germ cells and/or blood cells. Grüneberg concluded that the retarded development in early embryonic stages was the most fundamental known anomaly of the "flexed" mutant which is characterized by shortened and fused vertebrae, transitory anemia during liver hematopoiesis, and white regions on the belly and feet. Even the white regions of the C57BR strain can be traced to developmental anomalies in the lateral mesoderm

(Runner, 1954).

Auerbach's (1954) embryological investigations of "Splotch" (Sp) homozygotes in the mouse showed that abnormalities of the neural tube, including failure of closure in the hindbrain and lumbo-sacral regions, were responsible for the characteristics of kinky tail, spina bifida, cranioschisis; deficiency of neural crest derivatives and death during the 13th day of gestation. Neural crest tissue transplanted to chick coelom continued to develop, but melanocytes never appeared in the grafts. The pigment defect was limited to the neural crest; the retina was pigmented. There was a gradual decrease in the size of the dorsal root ganglia in an anteroposterior direction; ganglia in the lumbo-sacral region were usually missing. The correlation between the most severely affected region of the homozygote and the location of the ventral white "splotch" in the heterozygote suggests that there is a tendency to retardation of growth in the lumbo-sacral region even in the heterozygotes.

A lack of nerve cells in the colon wall is associated with a lack of pigment cells in the "piebald-lethal" (s^l) mouse (Green and Lane, 1962). The homozygotes are nearly white; they die from impaction and enlargement of the colon at 2-4 weeks of age. Green and Lane suggested that the action of the gene either reduces the proliferation of neural crest cells or prevents their migration.

Dunn and Mohr (1952) observed that anisocoria, as well as piebald spotting, was an effect of the "piebald" (s) gene in the mouse. They suggested that the abnormal development of the iris may be the result of a lack of migration of melanoblasts from the neural crest into the choroid.

A biochemical defect involving the metabolic activities of three types of cells (germ cells, melanoblasts and erythropoietic cells) was considered to be the most likely basis for the pleiotropic effects of the W and Sl mutants in the mouse (Billingham and Silvers, 1960; open discussion of Mintz, 1960). An almost complete failure of mitosis and retardation of migration of germ cells during the period of 9-12 days gestation was found by Mintz and Russell (1957) for W homozygotes. The few homozygotes which survive to maturity are sterile, anemic, black-eyed whites. Because the melanoblasts are migrating at the same time interval as the germ cells, it is likely that both types of cells are being affected in the same way.

The pleiotropic effects are generally less severe in W^V homozygotes. Males frequently are fertile at least on some genetic backgrounds (Mintz, 1960). Although proliferation and migration of germ cells is reduced, histological preparations of testes showed some tubules of normal spermatogenesis interspersed among empty ones. Apparently the normal regions represented clones of spermatogenic cells which had proliferated from the few germ cells that were able to complete migration to the germinal ridge. Homozygotes of W^V have unpigmented coats but do show some pigment in the skin of the ear tips (Silvers, 1956). Heterozygotes show some restriction of the pigment areas and absence of melanocytes in the unpigmented primary breaks.

The literature on the anomalies associated with piebald spotting in other mammals as well as the mouse is voluminous and cannot be reviewed at this time. Even the common piebald spotting of cattle is associated

with hypoplasia of the gonads in those animals which show the most white (Lagerlöf, 1956).

The pressure exerted by one melanocyte upon another in competing for space has been shown by Twitty (1949) and Twitty and Niu (1954) to be the most likely motivation for migration in both tissue culture and the living salamander. A single cell in tissue culture moved to the center of its confines; a group of cells spaced themselves equidistantly as though they were responding to metabolic gradients. Presumably mitosis indirectly induced migration by increasing the pressure of one melanocyte upon the other within the population.

The population pressure concept of melanoblast migration was essentially expressed by Russell and Major (1957) in their interpretation of the effects of in utero X-raying of C57BL mice at 10-1/4 days gestation. They found that the ventral white regions were significantly larger on the mice which were treated than on the controls. They concluded that some of the melanoblasts had been killed during their lateroventral migration and that the vacancies in the melanoblast population had been filled in by surviving cells. As a result the population did not extend to the ventral midline. This interpretation is in line with Willier's (1952) hypothesis that the population of melanoblasts has to be at the saturation point in order for the margin to be extended.

Schumann (1960) compared stained and silvered sections of mouse embryos from a piebald spotted (s) strain and the C57 strain. Pigment cells of the C57 strain were detected first in the dermis near the ear primordium in the 13-day-old embryos, but in the s strain they did not

become apparent until the 15th day. At 16 days pigment cells were identified in all regions except the legs and ventrum of the C57 mice. In contrast, pigment cells in the s strain were still limited primarily to the aural region. At 17 days the pigment cells of both strains moved into the epidermis and hair follicles. By the 19th day differentiation of the hair follicles and skin was completed. The C57 strain was essentially fully pigmented, but pigment cells were still absent in the forehead, ventrum distal half of the tail, and presumably the feet of the s strain. Thereafter no white region acquired pigment cells. Even the tactile hairs, produced by early differentiating follicles located just within the borders of the pigmented areas, remained white.

Schumann arrived at the following explanation for the development of the piebald spotting pattern in s mice. The primary action of the mutant gene is to retard the migration of the melanoblasts. Pigment cells can no longer migrate after the skin and hair follicles have differentiated. Hence, if the melanoblasts have not reached certain regions of the body by the time the skin and hair follicles have differentiated, those regions will remain void of melanocytes and grow only white hair.

Schumann was fortunate in being able to detect a distinct difference in the pigment cell distribution of the two stocks. Her material was far from being the most desirable. Not only were s and its wild-type allele on different genetic backgrounds, but the wild-type allele was in the C57 strain which is homozygous for another piebald spotting gene (Grüneberg, 1952; p. 75). Schumann showed the C57 embryos to be completely pigmented in her diagrams. It is doubtful that the feet, tail tip and mid-ventrum

were all completely pigmented; no examination of these specific regions was mentioned in the text. According to the diagrams, the feet of the s mutants supposedly became completely pigmented, but at least the front feet of an F_2 ss segregate are clearly white in a photograph in a later publication (Danneel and Schumann, 1961). If the s mutation had been compared with the wild-type allele on the same genetic background, the difference would likely have been even greater than that found by Schumann.

Evidently, Schumann (1960) was not able to identify melanoblasts until after they had been in a region for some time. She and Rawles (1947) both worked with the same strain (C57). Schumann did not identify pigment cells in the limb buds until the 17th day of gestation, but Rawles found that melanoblasts were present in grafts of limb buds taken from 12-day-old embryos. Although the delay of two days in the differentiation of melanoblasts into recognizable pigment cells was probably indicative of retarded migration in the s strain, Schumann's observations unfortunately cannot be used as proof.

Schumann failed to mention that pigment continues to spread in the tails of piebald spotted mice until they are two months old (Reynolds, 1954). Though she indicated that her explanation applied to piebald spotting patterns of all mammals, Schumann did not mention that pigment spread occurs in the skin around spots in guinea pigs and Holstein-Friesian cattle during postnatal growth (Billingham and Silvers, 1960). Nor did she account for the fact that the small, round, pigmented spots, characteristically found in the coats of Dalmatian and some hunting dogs,

do not appear until the pups are 3 weeks old even though a typical piebald spotting pattern is present at birth (Trimble and Keeler, 1938; Whitney, 1954; Little, 1957). Whitney (1954, p. 197) even describes a flecking pattern in hunting dogs which is not evident until the pups are 4 months old.

In reviewing the literature, it becomes apparent that the differentiation of the hair follicles, but not the differentiation of the skin in general, is closely correlated with the depletion of pigment cells in the epidermis. If so, Schumann's observation that the arrestment of melanoblast migration in piebald spotted mammals was correlated with the differentiation of the hair follicles has significance, but her conclusion that the concurrent differentiation of the skin set up a barrier to melanoblast migration probably is not valid.

In the mouse, it appears that the pigment cells in the epidermis are limited in number and are exhausted as soon as the hair follicles differentiate. In the tail, hair follicles are so scarce that the majority of melanoblasts are still free to migrate. Rawles (1947, 1955a) observed that the developing hair follicles have a very strong affinity for melanoblasts. Reynolds (1954) demonstrated by means of the dopa reaction that melanocytes were present in the epidermis of newborn mice. In the days following birth the melanocytes became more and more limited to the regions of the hair follicles. By the 12th day the melanocytes were confined to the matrices of the hair bulbs. In another phase of the investigation Reynolds treated the skin of agouti mice at 3 day intervals with a mixture of turpentine and acetone to induce hyperplasia; after

three months she was able to detect melanocytes in the epidermis again by means of the dopa reaction. Reynolds concluded that "non-pigmentary melanocytes" had been activated by the irritant. However, Szabó (1960) demonstrated in a similar experiment that the pigment cells, which were found in the dermis following treatment, had migrated out of the hair follicles. The evidence is strong that the pigment cells are normally limited to the hair follicles in the coated regions of the mouse although they may be present in the epidermis and dermis of regions of sparse hair covering such as in the tail and ears.

Epidermal melanocytes are present in the pigmented areas of the coats of piebald spotted guinea pigs and Holstein-Friesian cattle (Billingham and Silvers, 1960). A ring, in which the skin is pigmented but the hairs are white, is formed around each colored spot by an outward spreading of pigment. Probably the hairs in the ring remain white because the follicles differentiate before the pigment cells arrive. The reason for depletion of the epidermal melanocytes in the mouse but not in the guinea pig could possibly be related to the growth cycles of the hair follicles in the two species. Chase (1954) reviewed the hair growth cycles in mammals. In the mouse, development of the hair follicles is initiated in the period extending from 7 days before to 4 days after parturition. All follicles of one region pass through an active growth phase at one time followed by a period in which they all "rest". Conversely, in the guinea pig the growth period of the hair is longer and the cycle in one follicle is nearly independent of the cycles in neighboring follicles. Because a nearly constant growth stimulus is maintained

in the region of the follicles and the demands of the follicles upon the pigment cell population possibly extend over a relatively long period of time, it may be that the epidermal supply of pigment cells is less likely to be exhausted in the guinea pig than in the mouse. Reams (1963) described the PET strain of mice in which migration of the melanoblasts was much more extensive than in any other known strain. It would be of interest to determine whether or not piebald spotting mutations can permanently restrict pigment cell migration in PET mice and whether the difference from other strains is inherent within the pigment cells or the growth cycles of the hair follicles. The characteristic, no doubt, has a genetic basis.

Pigment spread in the dog apparently differs from that of either the mouse or the guinea pig. When pigment spread takes place relatively late, as in the formation of the small black spots on the Dalmatian, all of the hairs of the colored spot are fully pigmented up to the border (Trimble and Keeler, 1938). Lovell and Getty (1957) examined the hair follicles in pups at birth and regular age intervals up to 28 months. They found that the dog developed a compound follicle rather than a simple one like that found in mice and guinea pigs (Chase, 1954). Accessory follicles bud off the initially simple follicle until there are as many as 10 hairs emerging from a single external orifice in the 28 month-old dog. Apparently, the developing follicles are in a state that can receive migrating pigment cells during a long growth period. Possibly the rate at which the population of pigment cells is generated does not greatly exceed the requirements of the continuously forming follicles in piebald spotted

dogs; otherwise pigment spread would be much more extensive than it is.

Trimble and Keeler (1938) observed that the small, black spots on some of the segregates of a Dalmatian X Collie cross showed white hairs mixed with the black. Uric acid secretion, which was high in the parental Dalmatian breed, was again high in the segregates which had clear black spots but was normal in those which exhibited white hairs in the spots. Whether the correlation of the absence of white hairs in the spots and the high uric acid excretion was a physiological or a genetic linkage was not determined. Trimble and Keeler did show that the extreme restriction of the primary pigment areas, which is typical of Dalmatians, segregated independently from the recessive gene for high uric acid excretion. Little (1957) considered the extreme restriction pattern ($\underline{s}^{w}$) to be an allele of the white collar pattern ($\underline{s}^{i}$) which is found in Collies. The "Ticking" factor ($\underline{T}$), which controls the development of the small pigmented spots of Dalmatians, is a different gene but requires the white background of a piebald spotted type in order to be expressed. Thus, the Dalmatian pattern is determined by two genes, $\underline{s}^{w}$ and $\underline{T}$. Since $\underline{T}$ is dominant, Trimble and Keeler's backcrosses of the F_1 to the Dalmatian were not adequate to test the linkage of $\underline{T}$ and high uric acid excretion unless the presence of white hairs in the black spots distinguished $\underline{T}/+$ from $\underline{TT}$.

At least a working hypothesis for the development of piebald spotting patterns can be derived from the literature reviewed to this point. The migration of melanoblasts in piebald spotting mutants is retarded. The normal process of hair follicle differentiation arrests migration before

the melanoblasts have reached all regions of the skin. If a parallel can be drawn from the effects of the W alleles on germ cells, blood cells and melanoblasts in the mouse (Mintz, 1960), a lack of mitotic stimulus may be involved in the retardation of migration of melanoblasts as well as germ cells. The rate at which the border of the pigment cell population advances would be dependent on the rate of mitosis, since population pressure has been shown by Twitty (1949) to be the primary motive for migration of melanoblasts and Willier (1952) has concluded that the population density is maintained at saturation.

The idea of incomplete migration was first suggested by Chase (1939) and considered again by Silvers (1956, 1961), but two predominating concepts prevented the hypothesis from being seriously considered. One concept was that melanoblasts migrated to all regions of the skin during embryogenesis regardless of the genotype (Rawles, 1955a). The second concept, that amelanotic melanocytes existed in the white regions of piebald spotted animals, was becoming highly controversial by the time Billingham and Silvers (1960) wrote their review on mammalian melanocytes.

Rawles (1955a) discussed the histological evidence regarding piebald spotting patterns and concluded that no pigment cells existed in the white regions. No melanocytes could be found in the hair follicles of the white regions of piebald spotted mice, rats or rabbits. The dopa reaction failed to reveal any melanocytes in the epidermis of the white regions in piebald spotted guinea pigs and mice even when hyperplasia had been induced by an irritant.

Bogart and Ibsen (1937) reported observing pigment in the medulla

of hairs taken from the white regions of piebald spotted cattle. However, Shaklee (1948) showed that the medulla was dark only when viewed with transmitted light. By viewing the white hairs with reflected light, he determined that no pigment was present within them.

With proper staining techniques, branched cells can be demonstrated in the superficial epidermis of white regions (Billingham and Silvers, 1960). Billingham and his earlier coworkers concluded that amelanotic melanocytes were present in the epidermis and that the branched cells of the superficial layers were effete end products of the hypothetical melanocytes. They further proposed that pigment spread from the pigmented spots into the white regions by an "infection" process whereby a melanocyte induced an amelanotic dendritic cell to produce pigment through contact in some way.

Silvers (1957, 1958) offered three types of experimental evidence to disprove the infection hypothesis in favor of the pigment cell migration hypothesis for pigment spread. (1) In grafts between pigmented and non-pigmented regions, islands of pigmented hairs appeared which had no contact with cells in the pigmented areas. (2) In dilute (d) and leaden (ln) mutants of the mouse the morphology of the melanocytes differed from normal but the branched cells of the superficial epidermis remained normal; hence, the latter must not have been effete melanocytes. (3) Grafts deprived of the neural crest component still exhibited the branched cells; hence, the branched cells must not have been of the same origin as melanocytes.

Billingham and Silvers (1963) reported results of experiments which

they had conducted to determine whether the melanocytes in the ring around a pigmented graft on a nonpigmented host exhibited the immunogenic properties of the donor or host. All possible transplants including the reciprocals between two inbred lines of piebald spotted guinea pigs and their F_1 hybrids were performed. Pigment cells were transplanted from the pigmented spot of the donor to a white region in the host. Grafts between the inbred strains and from the hybrid to either parent were rejected but from inbred to hybrid were accepted as expected in the usual immunological response. Melanocytes taken from the ring of pigment spread around the graft in the F_1 host were accepted by the parental donor of the graft. Thus, the cells of the spread region had the same immunological properties as the graft donor and must have gone out into the ring by migration; that is, "infection" of F_1 cells did not take place.

Although Rawles (1955a) concluded that no pigment cells existed in the white regions of piebald spotted animals, she believed that they must have existed at one time but later moved out or degenerated. Her basic assumption that melanoblasts migrate to all regions of the skin during embryogenesis may be incorrect. Rawles (1947) did not transplant tissue from the mid ventrum, tail tip, or tips of the limb buds to determine whether or not melanoblasts ever reached those regions. If the piebald spotting pattern of the C57 strain exists in Rawles's subline, such grafts might prove to be void of pigment cells. Grafts of the neural crest of the all-white homozygotes of some piebald spotting mutants of the mouse have failed to produce melanocytes (Silvers, 1961), but no grafts have been made from the heterozygotes or other piebald mutants in which the

restriction of pigment areas is less severe. Rawles's (1955a) experiments involving transplantation of skin between the pigmented and nonpigmented regions of fetal and newborn hooded rats showed that there was no inhibition of pigment cells which migrated into the potentially white regions of fetal rat skin. Although it is still possible that the white regions do not offer an environment conducive to the differentiation or survival of melanoblasts, Rawles's own experiments show that the period unfavorable to survival would have to be very limited in duration.

If melanoblasts do not necessarily reach all regions of the skin in the piebald spotting types, it could be expected that the pigmented spots would correspond to some pattern of differential migration. The final effects can be correlated with what is known about migration of melanoblasts in many cases.

The melanoblasts do not migrate out at random from the neural crest but tend to follow preferential pathways (Willier, 1952). The melanoblasts which will pigment the skin and feathers migrate laterally and ventrally until they reach the boundary between the dermis and epidermis. The melanoblasts follow this interface as their population increases in size and spreads out over the body surface toward the midline of the ventrum. According to Fox's (1949) transplantation experiments on Barred Rock embryos, the proximal, dorsal skin is the first region of the limb bud to receive melanoblasts; from there they spread ventrally and distally over the limb bud. Watterson (1942) and Fox both noted that the wing tips and ventrum, which would be the last regions to receive melanoblasts, remained white in the down pattern of the Barred Rock.

Reams (1956) determined the pathways of migration taken by the melanoblasts which pigmented the coelomic lining of Black Minorca and Black Minorca - Golden Leghorn hybrids. Cross sections through the region of the umbilicus showed that the pigment cells of the abdominal coelomic lining, of the mesenchyme of the umbilical collar, and of the dermis of the ventral skin around the collar all belonged to one continuous population. It was evident that all of the melanocytes of the abdominal coelomic lining had migrated in from the neural crest via the ventral dermis. When the ventral dermis lacked pigment cells, there were never any in the abdominal coelomic lining. It was apparent that the thoracic region of the coelomic lining received its melanoblasts from the dermis overlying the lateral thoracic cage; again no pigment cells were present in the lining of the thoracic coelom if there were no pigment cells in the dermis covering the lateral thorax. In contrast, it appeared that the nephric region of the coelomic lining received its melanoblasts directly from the ventral component of the neural crest.

Landauer (1954) reported that the tendency toward pigment restriction was greatly amplified in Black Minorca embryos which were treated at 96 or 120 hours incubation with teratogenic compounds. The degree of restriction of pigmented areas and the severity of the induced morphological anomalies were correlated; pigment was restricted to the back in the most extreme cases. Effects were less pronounced when the embryos were treated at 24 hours incubation. Landauer suggested that the retarded migration of the melanoblasts was a result of selective mortality or reduced metabolic activity of the neural crest cells.

The effect of white wing tips and white ventrum in the down pattern of the Barred Rock (Watterson, 1942; Fox, 1949) and Black Minorca (Landauer, 1954; Reams, 1956) can be attributed to the mutation E (Hutt, 1949). Punnett and Pease (1927) demonstrated that an extreme piebald spotting pattern could be obtained in the down by combining another mutant with E. Regardless of whether the extreme pattern was derived by treatment or a combination of genetic factors, white feathers were replaced with pigmented ones in the adult plumage.

Characteristics of the growth cycles of feathers possibly determine whether or not piebald spotting patterns can permanently exist in a species of birds just as the growth cycles of the hair seem to affect pigment spread in mammals. Permanent piebald patterns are not found in chickens and turkeys but are found in pigeons and ducks (Hollander, 1944). Rawles (1960) reviewed the growth and molt cycles of feathers in different species. The plumage of those species which must fly upon leaving the nest is completed at an early age, but growth of feathers continues for a much longer period of time in the gallinaceous birds. Once the feather follicles mature, melanocytes can no longer be identified in the epidermis of the feathered regions of the chicken even though they may be present in the epidermis of unfeathered regions such as the feet and beak (Rawles, 1955a, 1960). The plumage of a chicken always includes feathers of two or more generations during the first 6 months (Stettenheim, 1961). The staggered maturation of feather follicles in the chicken may possibly allow for the existence of an epidermal population of melanoblasts until the migration of melanoblasts has progressed

completely to the tips of the wings. Thus, a gene for piebald spotting in the chicken could only produce a temporary pattern in the early growth phases. If, in non-gallinaceous species, the epidermal supply of melanoblasts is exhausted by the nearly simultaneous maturation of follicles, it may not be possible for melanoblasts to migrate after the growth of the first feather-coat is complete. If the action of a piebald spotting gene should retard the migration of melanoblasts, regions not already populated by pigment cells at the time of feather follicle maturation would remain void of melanocytes and grow only white feathers. Even piebald spotted ducks may not be an exception to the hypothesis. The juvenile feather-coat of the Mallard is completed before moulting begins (Millais, 1902).

The migratory paths of melanoblasts in the mouse are similar to those in the chick. Reams and Nichols (1959) found evidence that the lining of the coelom of PET mice became pigmented in three major areas (pleura, diaphragm and peritoneum) by migration of melanoblasts from the neural crest to separate sites of entry. In Mayer and Reams's (1962) experiments with the PET strain of mice, the melanoblasts had already entered the epidermis by the time they reached the hind limb bud early in the 12th day of gestation. No melanoblasts were included when grafts including ectoderm were transplanted from the hind limb bud to chick coelom at 11½ days; but at 12½ days melanoblasts were included. Grafts of pure mesoderm failed to yield melanocytes at both stages. Since migration of melanoblasts is much more extensive in the PET strain than in any other strain of mice, it is not surprising that they enter the

epidermis precociously. From the dorsal side of the base of the hind limb bud, migration of melanoblasts through the ectoderm was traced distally and ventrally. Where the margins of the melanoblast population met distally and ventrally, the melanoblasts made inroads on the mesoderm as evidenced by the presence of melanocytes in grafts of pure mesoderm transplanted at 14½ days gestation. Melanoblasts reached the ventral surface of the leg a day later than the dorsal surface, but the ventral pigment cells began to produce pigment first (at 16 days). Mayer and Reams concluded that the time of differentiation of melanoblasts in different areas was not directly related to their arrival time. Their conclusion renders Schumann's (1960) criterion for detecting pigment cell migration quite unsatisfactory.

Most determinations of the presumed paths of melanoblast migration in the skin of mammals have been made through the observations of piebald spotting patterns in the coat. Lauprecht (1926) confirmed Allen's (1914) analysis of pigment spread in cattle except that he included an area for the eye and cheek which Allen had considered as a possibility. Burn's (1952) diagram of the pigment centers for the dog differed from Allen's description in that the pleural center was divided into two separate centers, the sacral was single and medial instead of bilaterally paired, and a single medial caudal center was added. Charles (1938) revised the centers in the mouse to include eye, ear, scapular, mid-dorsal, lumbar and rump centers on each side.

Mosaic coat color patterns of $\underline{Mi}^{wh}/+$ mice were utilized by Schaible (1959) and Schaible and Gowen (1960) to trace the spreading out of

melanoblasts from the pigment centers (Plate 1). Repeated occurrences of each pigment area showing wild-type instead of the dilution effect of the heterozygote demonstrated that all of the pigment cells of an area were proliferated from a single melanoblast in which a genetic change had taken place. By sketching the location of the altered areas on standard outlines, it was possible to map the pigment areas of the entire body surface (Fig. 7). Fourteen primary pigment areas were demonstrated: six bilaterally paired (nasal, temporal, aural, costal, lumbar and sacral) and two medial (coronal and caudal).

Certain piebald spotting mutations restrict the size of specific pigment areas. The mutant described by Goodale (1937) initially showed only a few white hairs in the coronal area. Pullig (1949) described a spotting pattern in which the temporal areas were absent; she attributed the effect to a new allele of s. Pigmentation of the caudal area is restricted by a mutant gene in the C57BL strain (Grüneberg, 1952). Schaible (1959) observed that the belt (bt) mutation primarily restricted the lumbar centers. Selection for more white in the lumbar areas resulted in the complete loss of the pigment centers and restriction of adjacent pigment areas.

Although the examples are far less numerous, physical barriers as well as reduced mitotic activity can restrict the migration of melanoblasts. Entrance ways to specific regions or even the entirety of the epidermis may be blocked to invasion of melanoblasts. The resulting void of melanocytes in the epidermis constitutes a piebald spotting pattern.

Dalton (1950, 1953) concluded from his transplantation experiments

Plate 1. $\underline{Mi}^{wh}/+$ mosaics

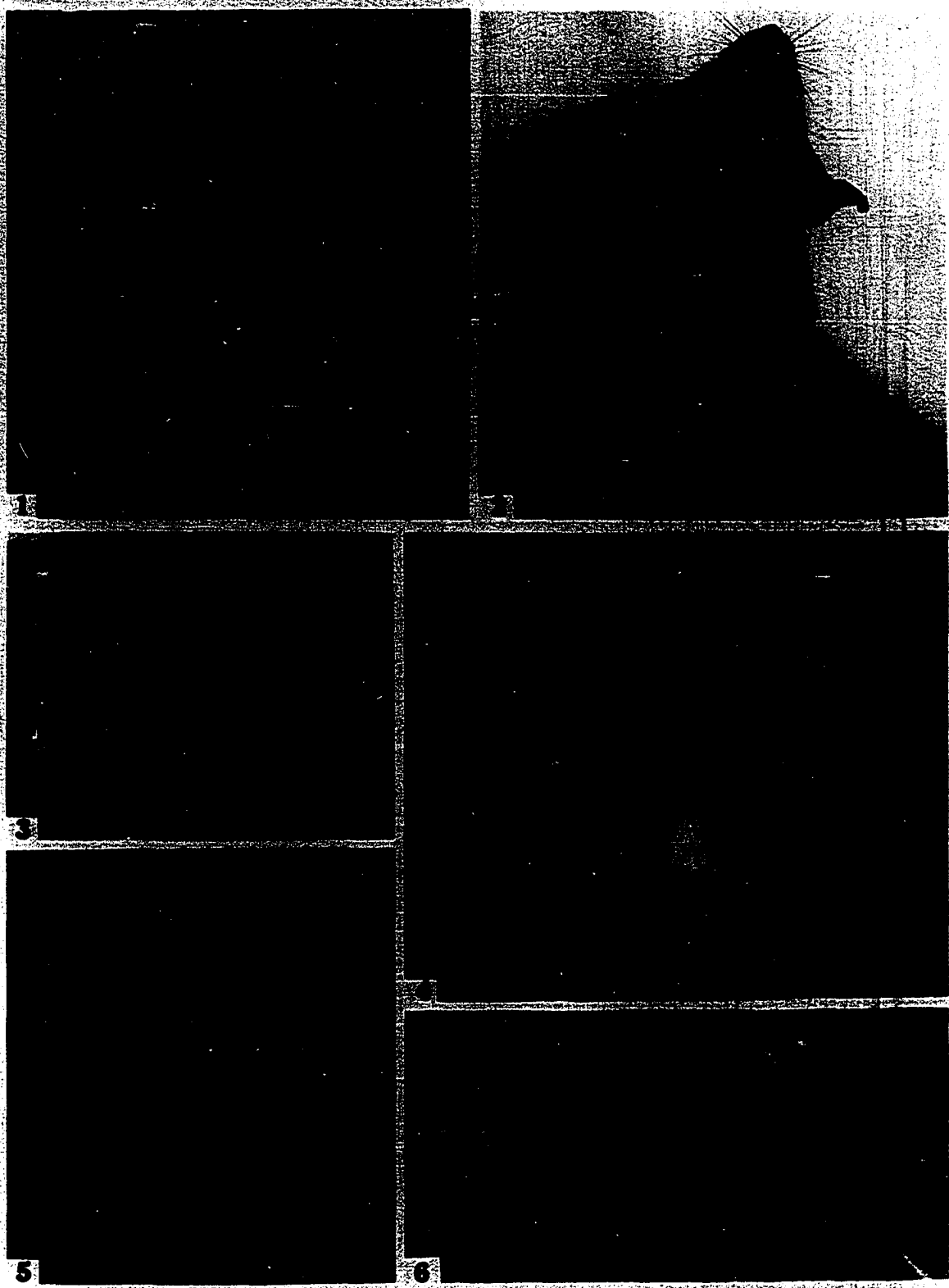
Figs. 1 and 2. Dorsal and ventral views of a right costal mosaic

Non-$\underline{Mi}^{wh}$ melanocytes are presumed to have also migrated to the left side as a result of competition with $\underline{Mi}^{wh}/+$ melanocytes.

Fig. 3. Coronal and right nasal non-$\underline{Mi}^{wh}$ areas

Fig. 4. Left lumbar and restricted right costal non-$\underline{Mi}^{wh}$ areas on $\underline{a}^{t}\underline{a}$ $\underline{bt}/+$ $\underline{Mi}^{wh}/+$ and $\underline{a}^{t}\underline{a}$ $\underline{btbt}$ $\underline{Mi}^{wh}/+$ genotypes respectively

Fig. 5 and 6. Lateral views of the same lumbar mosaic shown in Fig. 4 above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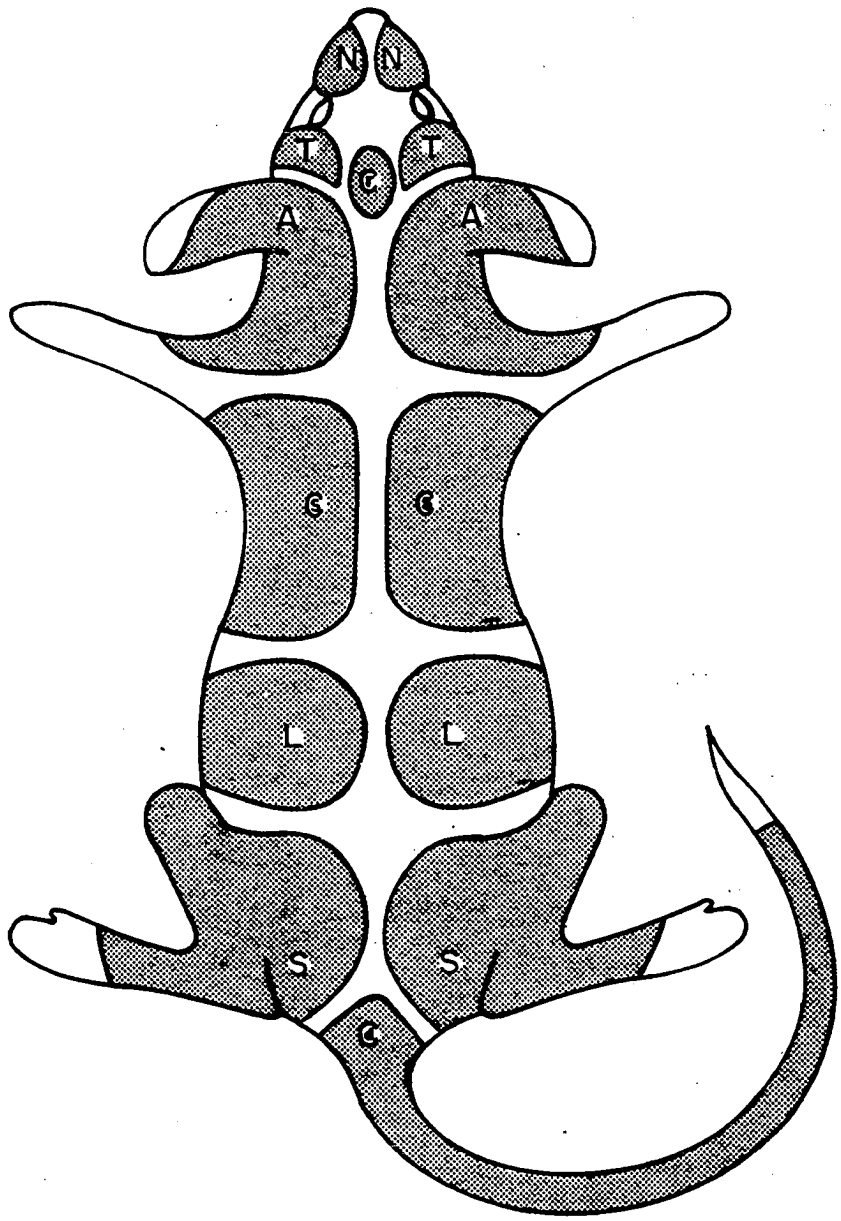


BILATERAL CENTERS

N NASAL
T TEMPORAL
A AURAL
G COSTAL
L LUMBAR
S SACRAL

MEDIAL CENTERS

C CORONAL
c CAUDAL

Symbols mark the location of centers

Fig. 7. Pigment areas and centers in the mouse

that melanoblasts were blocked from entering the epidermis of the white axolotl which is a recessive mutant of the black type, Siredon mexicanum. Neural crest of either type would produce well differentiated melanocytes full of pigment in explants or transplants to the midventral belly region of either black or white host embryos. The epidermis of black embryos appeared to provide a pathway for migration of melanoblasts from the neural crest to the belly while the white epidermis was not conducive to melanoblast migration. Narrow rectangular strips of epidermis were transplanted from black to white embryos prior to migration of melanoblasts. Strips placed vertically on the flank became fully pigmented, but those placed horizontally at the same level as the lower end of the vertical strips remained unpigmented. In the white axolotl, pigment cells accumulated beneath the unpigmented dorsal epidermis; apparently the melanoblasts could not take an alternate route through the mesenchyme.

Reams (1956) found no melanocytes in the lining of the abdominal coelom of Black Minorca chicks when there were no pigment cells in the dermis of the skin surrounding the umbilicus. If the melanoblasts had not yet migrated into the abdominal coelom by the time of hatching, the rearrangement of the mesenchymal cells at the umbilical collar apparently formed a permanent block to pigment cell migration (Ris, 1941).

Watterson (1942) observed that melanoblasts could enter the epidermis in the Barred Rock embryo only during a very limited time, 80 to 91 hours incubation. Apparently, the number of melanoblasts which were able to gain entrance to the epidermis was insufficient to populate all of the follicles out to the wing tip in the down pattern. The down feathers on

on the wing tip were white even though pigment cells had migrated out that far in the dermis. Reams (1956) observed that only unbranched melanoblasts could invade a feather papilla. In his grafts of coelomic lining to the limb bud of a host embryo the unbranched melanoblasts entered the down follicles, but the branched melanocytes remained in the dermis.

Other mutants exhibiting white regions of the coat

In contrast to the piebald spotting patterns in which no pigment cells of any kind can be detected in the white regions, there are mutant types in which pigment cells can be found in the white as well as colored regions.

Albinism and the "Himalayan" effect of an allelic gene in some mammals was reviewed by Billingham and Silvers (1960). In the hair follicles of albinos there are cells which correspond in position and morphology to the melanocytes of pigmented mammals. A defect in the synthesis of tyrosinase renders the potential melanocytes incapable of pigment production in the albino. That the "clear cells" in the hair follicles of albinos really are amelanotic melanocytes, has been demonstrated in a number of ways. When tissues from albino embryos were transplanted to the spleens of adult hosts prior to melanoblast migration from the neural crest in the donor, clear cells could not be found in the grafts; after melanoblast migration had taken place in the donor, clear cells could be found in the grafts. Pigment cells of resting follicles of pigmented animals are known to be selectively destroyed by X-irradiation; Quevedo (1957) found that the clear cells of albinos showed the same

sensitivity. In the Himalayan rabbit and similar mutants of the mouse and guinea pig only the extremities (ears, feet and nose) are ordinarily pigmented. The white regions can be made to grow pigmented hair by experimentally lowering the skin temperature. Thus, the white regions of the Himalayan types possess amelanotic melanocytes in contrast to a complete lack of clear cells in the white regions of piebald spotting types. The Himalayans are true partial albinos. Piebald spotting types have been erroneously referred to as partial albinos, particularly in the literature of human genetics, on many occasions (Cooke, 1952).

The stages in the development of color patterns in goldfish were described by Fukui (1927). The young goldfish has an olive drab color due to the presence of both melanophores and xanthophores. In most domestic varieties the melanophores begin degeneration on the belly and base of the fins. The process of melanophore degeneration spreads upward on the body and distally on the fins. At one stage the back is black and the belly is yellow. Depigmentation continues until only xanthophores remain, a gold fish being the result. In time the process may be repeated on the xanthophores. At one stage the back is gold and the belly unpigmented. Finally the fish may become pure white.

White goldfish resemble "Himalayan" mutants in that they produce pigment on the extremities and dorsum when exposed to cold temperatures. It is unlikely that amelanotic pigment cells are present in the white regions as in Himalayan types. When Goodrich and Nichols (1933) transplanted scales from pigmented to white regions, melanophores spread out into the unpigmented regions. When a white scale was transplanted to a

pigmented region, melanophores migrated into the grafted scale. If the white region were saturated with amelanotic melanophores, very little migration should take place (Rawles, 1955a,b).

Thus, white regions can form as the result of degeneration of melanoblasts which have already migrated to all parts of the body. Since the pattern is quite different from piebald spotting types, degeneration of pigment cells in the goldfish cannot be used as support for Rawles's (1955a) hypothesis that pigment cells degenerate in the white regions of piebald spotted animals. Possibly the white regions in the goldfish are not completely free of melanoblasts since production of pigment is possible when the temperature is lowered.

Melanocytes are present in the feather follicles of both the red and the white regions of the pile pattern of "dominant white" (I) chickens. The wild-type chicken exhibits both black and red pigment in the feather coat. In the red and white pattern, the black pigment is inhibited by I (Jaap and Hollander, 1954). In addition to I, most strains of White Leghorns carry E which eliminates red pigment; the resulting phenotype is completely white. Hamilton (1940) observed that early degeneration of the melanocytes in the feather follicles was responsible for white in breeds carrying the gene I or recessive white (c). Melanocytes occurred consistently in the regenerating feather germs of dominant white breeds but were less viable and appeared inconsistently in the follicles of recessive white breeds.

Genetic Mechanisms of Mosaicism in Relation to Cell Lineage

General considerations and terminology

Hannah-Alava (1960) reviewed mosaicism of plants and animals. More specialized reviews included mosaicism of Drosophila (Morgan, 1929); mammals (Carter, 1952; Robinson, 1957), birds (Hollander, 1944), and chickens (Cock, 1955) and the relationships of mosaicism to cancer (Schultz, 1959) and sex determination (Gowen, 1961).

Genetic mosaics are made up of two kinds of tissue, each expressing a contrasting phenotype determined by different alleles or chromosome numbers. The differences arise through anomalies of fertilization or genetic changes involving mutation or chromosomal aberrations.

It is almost imperative that differentiation of cell lines be considered along with the mechanisms of mosaicism. The number of cell lines affected reflects the stage at which the genetic change took place. When the change is germinal as well as somatic, additional information regarding the nature of the genetic change can be obtained through breeding tests.

Mechanisms will be reviewed in ontogenic order: (1) fertilization, (2) cleavage and early embryogenesis, and (3) proliferation of somatic cell lines. These developmental stages in many cases do coincide with times when genetic change can effect mosaicism. Demerec (1941) found that some mutants were unstable in all stages of ontogeny while others mutated only during maturation divisions, early embryogenesis, or late stages of cell proliferation.

Adequate operational terms and definitions must be specified in

some cases before the genetic mechanisms involved in the development of mosaicism can be consistently interpreted in either the discussion of literature or the results of the present study.

The distinction between mutation and chromosomal aberration is almost forced to be arbitrary since rearrangements can often be demonstrated within what was considered to be a locus at one time. Evidence for the chromosomal abnormalities is dependent on cytological observations or the following of genetically marked chromosomes. Neither of these methods is possible in many mammals and birds. There are two situations in which it seems necessary to make basic assumptions. (1) When there is good evidence that an individual began development as a homozygous or hemizygous type but exhibits areas expressing another allele, it will be assumed that a mutation occurred during ontogeny. (2) If the individual began development as a heterozygote and exhibits areas expressing the recessive allele, it will be assumed that the dominant allele has been lost, replaced, or inactivated in some way. The burden of proof should rest mainly on those who maintain that mutation has not occurred in the first case and that mutation has occurred in the second case. Although not always stated, these assumptions underlie the interpretation of most mutation studies in the literature. A review of some of those studies will illustrate the points.

Frequencies of "mutation" may be estimated by the specific locus test. The wild-type is treated and then bred to a mate that is homozygous for recessive markers at a number of specific loci. The frequencies of progeny showing a mutant phenotype of one of the specific loci are

recorded. Estimates of "mutation" frequencies obtained from specific locus tests are generally considered to be far less reliable than those obtained by tests of the hemizygous genes on the sex chromosomes. Ward and Alexander (1957) cytologically examined the "mutations" induced in 8 specific loci of the 3rd chromosome of Drosophila melanogaster. Twenty-six out of 56 cases induced by irradiation of mature sperm were associated with cytologically detectable chromosomal aberrations. Hence, proof of mutation seems imperative when dealing with heterozygotes.

The so-called unstable or mutable genes of Drosophila, Delphinium, and maize (Demerec, 1941) provide good examples for the first assumption given above. Because the genetic changes in most cases were from the homozygous recessive to a dominant allele, the changes were interpreted strictly as mutations for many years.

McClintock (1956, 1958) and Peterson (1961) described several two-factor systems each of which controls the mutability of several different loci in maize. Both factors can move from one site to another among the chromosomes of a cell. One has an inhibiting effect on the action of the dominant gene at the locus in which it resides and may be referred to as a suppressing element. The other factor controls the transposition of the suppressing element away from the locus and may be referred to as a controlling element. For example, if the suppressing element becomes attached to the A_1 gene of an A_1/a_1 heterozygote, the action of the dominant gene is suppressed to the point that only the expression of the recessive gene, colorless aleurone, is the result. If the controlling element is also present in the same cell, it is possible for the

suppressing element to be transposed to another position; whereupon A_1 is no longer suppressed and a fully pigmented dot or sector is formed. If the process always gave this result, it would appear that no actual change in the gene (mutation) was taking place. However, the suppressing element may assume different states. Suppression may not always proceed to the same level. Reversion may not proceed to the initial state of the suppressed gene and may characteristically occur at different stages of ontogeny. If pigmentation of the aleurone is the characteristic involved, the grade of color may be some shade in between colorless and red; the size of the dark areas may be some grade between fine dots and large sectors. If the controlling element is lost from the chromosome complement by segregation at meiosis or is excluded from a sector of a plant, the new shade of pigmentation will be faithfully reproduced by the new allele contained in the gametes derived from the altered tissue. Homozygotes of the new allele will be unstable if the controlling element is returned to the chromosome complement or stable if it is not. If only one controlling element is present, homozygotes segregate 3 mutable to 1 stable type when selfed. If the controlling element becomes attached to the gene along with the suppressing element, then the whole complex segregates as a mutable allele. Even though suppression of the action of a dominant allele instead of mutation could be the explanation for unstable recessive types, it appears that each effect is one of a graded series of suppressions that are possible. Since each grade can be reproduced as a new type, all levels may be considered to be mutations.

Two situations have been reported in which mutation appears to be

occurring in heterozygotes. As noted in the second assumption above, rigorous proof is required for the explanation that mutation is actually taking place.

Green (1960) reviewed cases in Drosophila in which genes were stable in homozygotes or hemizygotes but reverted to wild type during meiosis without benefit of crossing over in the heterozygotes. The phenomenon, referred to as "gene conversion", has only been reported in meiosis but mosaics could possibly arise by a gene converting its allele in the soma.

In reviewing his work with the R locus in maize, Brink (1960) used the term "paramutation" which he coined to denote the process by which one allele induces the other to produce a phenotype which is intermediate in effect. The paramutant (modified) allele never regains its original effect in subsequent generations. Paramutation can take place in early embryos; tassels have been found which are mosaic for different alleles.

The R alleles appear to constitute a complex locus. Each allele determines a different combination of several alternative characteristics: (1) green versus red seedlings, (2) colorless versus red crown, red stippled, or self red aleurone color, (3) paramutagenic versus non-paramutagenic. An inbred strain is used as a testcross parent; its characteristics (green seedlings, colorless aleurone, nonparamutagenic) may be considered as an operational standard of reference for the contrasting dominant characteristics. The loci within the complex have not been mapped, but some recombinants have been derived from known types. For example, Ashman (1960) obtained 18 colorless aleurone kernels in 30,491 testcross progeny of R^{st} (stippled and paramutagenic)/R^{r} (red

aleurone and red seedling) heterozygotes. In 5 of the 18 new types, recombination of the other characteristics of the complex (red seedling and paramutagenic) accompanied the loss of aleurone color. Eight were paramutagenic only. Five showed only the red seedling deviation from standard. It would be of interest to know what happened to the mutability factor which gives rise to the stippled characteristic. If the mutability factor is not allelic to the factor for self red aleurone, it could presumably become hypostatic upon crossing over. Or it may be a controlling or suppressing element which has transposed.

Although instability or controlled mutation of the gene seems to be a realistic interpretation of variegation effects in some cases, there are processes of meiosis (reduction, crossing-over and nondisjunction) which should be given serious consideration as possible mechanisms which result in the segregation of genes during mitosis in heterozygous individuals (Carter, 1952). However, the terms, "nondisjunction" and "crossing-over", have proved to be unsatisfactory at times when used to describe similar phenomena in mitosis.

Failure of chromosomal units to separate and pass into the daughter cells during cell division was termed "non-disjunction" by Bridges (1915). Nondisjunction of homologues during the reductional division of meiosis was termed "reductional non-disjunction". Nondisjunction of sister chromatids during the equational division of meiosis was termed "equational non-disjunction". There is no doubt as to whether homologues or sister chromatids are failing to separate when the term "nondisjunction" is preceded by the adjectives, "reductional" or "equational". However,

Bridges extended the meaning of the term to include failure of sister chromatids to separate during mitosis without giving any indication in the term "somatic non-disjunction" that sister chromatids rather than homologues were involved. Nondisjunction of sister chromatids in mitosis is equivalent to the same anomaly in the equational division of meiosis except that in mitosis the relationship of chromosomal elements is complicated by the diploid condition of the cell at that stage of cell division.

Even though pairing of homologues does not take place in mitosis to the extent that it does in meiosis, the passing of each homologue of a pair to a separate daughter cell can be considered as "mitotic disjunction". Nondisjunction of homologues is normally expected during mitosis since there is no reduction division; that is, disjunction rather than nondisjunction of homologues would be an anomaly in mitosis. In contrast, disjunction of sister chromatids is normal in mitosis; nondisjunction of sister chromatids is the anomaly.

As long as the normal behavior of the chromosomal elements is used as a standard of reference, as it will be in this thesis, there should be no confusion. When nondisjunction is referred to as an anomaly in mitosis, it follows that the sister chromatids of one homologue are failing to separate; the daughter cells are monosomic and trisomic. When disjunction is referred to as an anomaly in mitosis, it can be assumed that one homologue of a pair is passing into one daughter cell and the other into the other daughter cell.

No reduction of chromosome number is implied for mitotic disjunction.

Separation of sister chromatids is only delayed until after cell division is effectively completed. It may be that disjunction of homologues is the normal reaction to be expected in mitosis if the separation of sister chromatids is delayed. The following experiment will illustrate some of the points just made.

Mickey and Blount (1952) observed mosaics in the offspring of Drosophila that had been treated in the larval stage with vapor of glacial acetic acid. They concluded that the sister chromatids in both homologous chromosomes failed to separate at the normal time and that the homologous chromosomes segregated to opposite poles during mitosis of some somatic cells. This phenomenon they termed "somatic disjunction". Because all of the recessive markers of the 3rd chromosome (ru h st p^p ss e^s) were expressed in the mosaic area, they concluded that somatic crossing-over could not have occurred except for a very unlikely double crossover in the 4 units between st and p^p, crossovers being on each side of the centromere. The mutant tissue was viable; hence, the sister chromatids must have maintained the diploid state of the 3rd chromosome.

The term "somatic non-disjunction" is unsatisfactory when applied to mitoses of germ cells, cleavage cells, or the totipotent cells of plants or animals which can regenerate germinal as well as somatic tissue. Investigators in the field of human genetics distinguish between "meiotic non-disjunction" and "mitotic non-disjunction" in discussing the origins of various heteroploid types and mosaics thereof (Stewart, 1962). In this thesis, the adjectives (mitotic, germinal and somatic) will be used depending upon the presumed state of cell differentiation.

Stern (1936) reported that the repulsion heterozygotes for two recessive markers in Drosophila often exhibited spots in which one of the recessive mutants was expressed or else twin spots in which a different marker was expressed in each spot. He interpreted the effect as being due to somatic crossing over involving two strands in a four strand group. Even though Stern took extreme care to point out that no reduction of homologues was involved and that the segregation of genes could be expected as the result of otherwise normal mitosis, the phenomenon is often misconstrued as being essentially meiosis in the soma. For example, Sinnott et al. (1958, p. 175) state: "But if a cell of this constitution, + sn/y +, undergoes a meiosislike process, cells homozygous for y and others homozygous for sn will be produced."

As in the case of nondisjunction, the qualifying adjective, "somatic", is again too restricting for discussion of crossing over during mitosis in general. For example, Russell and Major (1956) referred to genetic changes that resulted in mosaicism of both germinal and somatic tissues as being "due to somatic, rather than germinal, events". Undoubtedly, the distinction was being made that the genetic change was taking place in the cells of the early embryo rather than in the gametes of the parents. Perhaps confusion can be avoided in this thesis if the term "mitotic crossing over" is used when referring to mitosis of cells in general and the adjectives, "somatic" and "germinal" are reserved for reference to cells which presumably have differentiated one way or the other. It follows then that germinal crossing over can give rise to a cluster of recombinant progeny just as somatic crossing over can give

rise to tissues that are a mosaic of genotypes.

Fertilization

Certain characteristics of the fertilization and sex determination processes in the parasitic wasp, Habrobracon juglandis, render it particularly subject to mosaicism (Whiting, 1943; Whiting, 1961). Males ordinarily are haploid, arising from unfertilized eggs. Fertilized eggs become females if they are heterozygous for the sex alleles but become males with low viability when homozygous. The homozygous diploid male tissue is more likely to be sustained if it is incorporated with haploid male tissue or with heterozygous female tissue in a mosaic. Impaternate diploid females can be produced when patches of ovarian tissue become tetraploid and undergo meiosis. In general, binucleate eggs are produced about one percent of the time; there is evidence that trinucleate eggs can also occur. About one percent of all eggs are fertilized by two sperms (dispermy). Combinations of these peculiarities give rise to certain types of mosaics.

Some mosaics arise without fertilization; binucleate or trinucleate eggs develop into haploid mosaic males. One male arose from a trinucleate egg in which each nucleus represented one chromatid from a tetrad in which four markers were involved. The testes were found to be stratified for cells proliferated from two of the nuclei. The male bred as a different genotype alternately in three successive cultures. Impaternate gynandromorphs are formed from binucleate eggs in which one nucleus is haploid and the other is diploid; the female nucleus evidently arises in the same manner as impaternate females.

The variety of mosaics arising from fertilized eggs is, of course, much greater. Fertilization of one nucleus of a binucleate egg and gynogenesis of the other results in a gynandromorph or a haplo-diploid male mosaic. Dispermic fertilization of both nuclei results in a mosaic female if both parts are heterozygous for the sex alleles or a gynandromorph if one part is homozygous male and the other part heterozygous female. Or a trinucleate gynandromorph can develop as a result of one nucleus being fertilized by one sperm, the other egg nucleus undergoing gynogenesis, and a second sperm undergoing androgenesis. In dispermic fertilization of a normal egg, the supernumerary sperm may undergo androgenesis to form a gynandromorph if the fertilized egg is heterozygous for the sex alleles or a haplo-diploid mosaic male if the fertilized egg is homozygous.

Similar mechanisms have been found in other species. Androgenesis is believed to be a cause of mosaicism in pigeons (Hollander, 1949), chickens (Cock, 1955, 1959) and honey bees (Rothenbuhler, 1958). Abnormal meiosis in unfertilized eggs of the honey bee can result in mosaic males formed from the development of two haploid pronuclei bearing contrasting genetic factors (Tucker, 1958). A gynandromorph results if the two pronuclei cleave and two of the daughter nuclei unite; female tissue develops from the resulting diploid nucleus and mosaic male tissue from the haploid nuclei. Dispermic fertilization of binucleate eggs has been known to produce mosaics in Drosophila melanogaster and silkworms (Goldschmidt, 1937). Fusion of male and female zygotes has been offered as a possible mechanism for the occurrence of gynandromorphs in mice

(Hollander et al., 1956). Gynandromorphic mice have since been produced experimentally by this means (Tarkowski, 1962).

Cleavage and early embryogenesis

From the examination of mosaic individuals, theoretically it should be possible to determine when different cell lines become permanently separated during ontogeny (Morgan and Bridges, 1919; Muller, 1920; Mohr, 1923). If two or more cell lines show characteristics which are different from the phenotype or known genotype of the rest of the body, then it may be assumed that a genetic change occurred in an early stem cell which gave rise to all of the altered cell lines. If, on the other hand, two or more cell lines include both altered and unaltered phenotypes, then it can be assumed that the cells of each of those lines descended from more than one stem cell. Finally, if a genetic change (which is known to affect characteristics of more than one cell line) is reflected in only one of them, the change must have taken place after the cell lines became separated.

Generally, a gene affects only one tissue; the effect may be quite localized as in the case of eye color in Drosophila. In mosaics of such mutants, genetic changes in only two cell lines can be detected, the line affected by the mutant and possibly the germ cell line. The germ cell line is the only line in which genetic mosaicism can be detected when there is no actual change in the phenotype of the cells. If mosaicism is caused by a chromosomal anomaly involving several marked loci, then genetic changes can be detected in many parts of the body even though each gene may affect only one characteristic (Bonnier et al., 1949).

The relationship of cell lines in respect to their time of separation may be an important factor in determining what cell lines will be affected by genes with pleiotropic effects. If so, mosaics of mutants which exhibit pleiotropic effects should be especially good material for the study of separation of cell lines. Theoretically, a genetic change involving a pleiotropic gene could affect several cell lines if the change occurred early enough in ontogeny. Three mutants of the mouse ($\underline{W}^a$, $\underline{Mi}^{wh}$, and $\underline{Va}$) show promise of being excellent material for such studies in that they all three exhibit pleiotropic effects and frequently mosaicism of coat color (Schaible, 1959; Schaible and Gowen, 1960). The $\underline{W}$ alleles have been studied extensively (Mintz, 1960), but the possibility that mosaicism of the blood or germ cell lines might be a contributing factor to the viability or fertility of exceptional homozygotes does not seem to have been considered.

Carter (1952) reported a $\underline{W}^V$ heterozygous female that was mosaic for both coat color and germ cells. The aural, lumbar and sacral areas on the right side, the tail, and extensions to the left side of the aural and lumbar areas were wild-type as contrasted to the dilute color of the rest of the coat. When mated to wild-type, the mosaic produced 31 heterozygous and 10 wild-type progeny.

Carter discussed the possible causes of mosaicism in relation to the formation of somatic and germinal cell lines. In regard to the mosaic described above, he suggested that one gonad was genetically different and was producing only $\underline{W}^V$ gametes while the other was producing the expected ratio of 1 $\underline{W}^V$: 1 +. Mintz and Russell (1957) later observed that

the migration and proliferation of $\underline{W}^{V}\underline{W}^{V}$ germ cells was greatly retarded beginning at the 9th day postfertilization and that most of them perished before reaching the germinal ridge. All mechanisms considered by Carter, except for mitotic nondisjunction, would require survival of $\underline{W}^{V}\underline{W}^{V}$, $\underline{W}^{V}/0$, or $\underline{W}^{V}$ germ cells. Survival of an ordinarily lethal homozygous type often is not possible in Drosophila even when it segregates out in tissues of the heterozygote (reviews by Stern, 1955, 1958). If such were the case in the $\underline{W}^{V}/+$ mosaic mouse, mitotic nondisjunction would be the most likely explanation; the abnormal cells would carry at least one wild-type allele ($\underline{W}^{V}/\underline{W}^{V}/+$). On the other hand, occasional $\underline{W}^{V}$ homozygotes are partially fertile (Grüneberg, 1952; Mintz, 1960); hence, it would not be impossible for $\underline{W}^{V}\underline{W}^{V}$, $\underline{W}^{V}/0$, or $\underline{W}^{V}$ tissues to produce gametes.

Mitotic nondisjunction of autosome 21 in the second or later cleavage division was the explanation offered by Fitzgerald and Lycette (1961) to account for a human mosaic whose cultured leucocytes showed counts of 46, 47, and 48 chromosomes. The individual had some mongoloid features and was presumed to have arisen from a trisomic zygote. As precedents, Fitzgerald and Lycette referred to another case of mosaicism for chromosome 21 in man and an X-XX-XXX gynandromorph in Drosophila. Stern (1960) reported the gynandromorph as probably arising by mitotic nondisjunction in a female of normal chromosome constitution.

From Carter's (1952) diagrams and Figs. 8-10 of this thesis, it can be seen that at least two stem cells must contribute to both the germplasm and soma in order for a single genetic change to produce mosaicism in two cell lines as, for example, in the germ and pigment cell lines of the

$\underline{W}^{v}/+$ mosaic which Carter reported. But if each cell line should be descended from a single stem cell, as in Fig. 8, a single event of mitotic crossing-over, for example, could only produce two homogeneous cell lines, $\underline{W}^{v}\underline{W}^{v}$ and ++. One event of mitotic nondisjunction could contribute $\underline{W}^{v}/\underline{W}^{v}/+$ to the daughter cell which gives rise to the germ cell line and +/0 to the daughter cell which joins the other previously segregated $\underline{W}^{v}/+$ somatic cells. Although the germinal tissue would appear to be mosaic in a progeny test, the entire pigment cell line would have to be +/0 or $\underline{W}^{v}/+$. Thus in all cases, two events of genetic change would be necessary to produce mosaicism in two cell lines if each line really descends from only one stem cell. On the basis that two events would be infrequent, Carter ruled out mutation and deletion and favored mitotic reduction and mitotic crossing-over as the most likely mechanisms of mosaicism. The validity of his argument is at least partially dependent upon the question of whether or not all cells of a line are derived from only one stem cell. Since a considerable amount of work has been done on the lineage of germ cells, it seems worthwhile to review what is known.

Tyler (1955) and Burns (1955) reviewed the origin of germ cells. The germ cell line and the undifferentiated cell line from which it is derived can be identified from the first cleavage throughout ontogeny in a few species. The definitive cell which gives rise exclusively to the germ cell line is not formed until cleavage III in Miastor and IV in Ascaris. In prior cleavages one daughter cell becomes somatic and the other remains undifferentiated (Fig. 8). The undifferentiated cells and the germ cells can be distinguished from the somatic cells by such

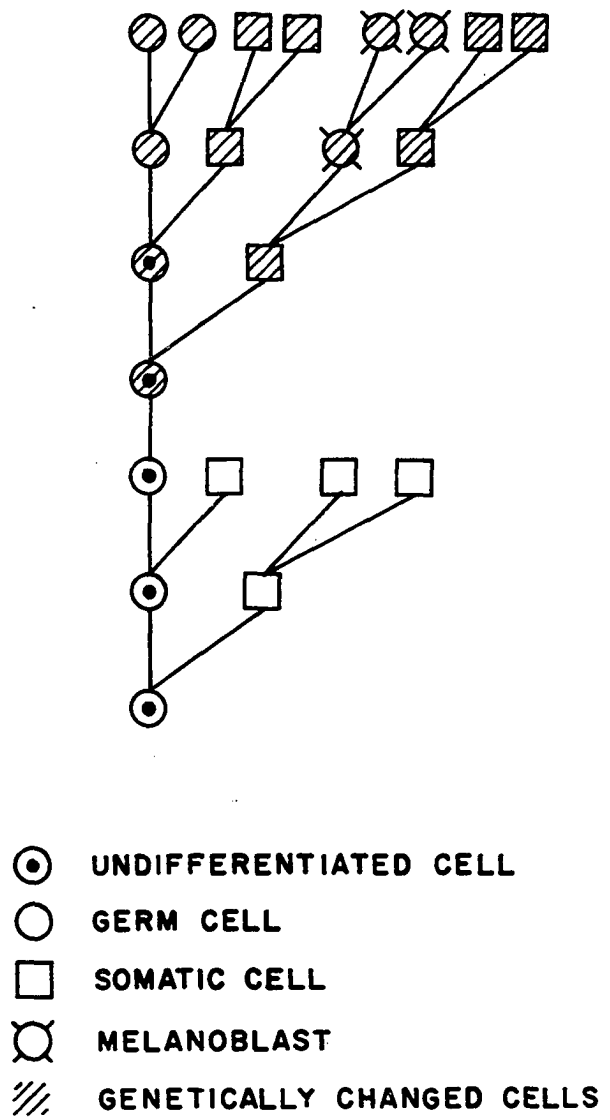


Fig. 8. Derivation of the entire germ cell line from a single stem cell

A single event changing the genotype of a blastomere will result in a homogeneous genotype in the germ cell line and any other cell line which descends from that single stem cell.

morphological characteristics as larger size, retention of characteristic cytoplasm (poleplasm), and undiminished nuclei.

In Tisbe, a small crustacean, the early cleavage divisions are similar to Ascaris and Miastor except that two like daughter cells are produced from the undifferentiated cell in cleavage VI (Witschi, 1934). Each daughter cell divides again to form one germ cell and one entodermal cell. Each of the germ cells moves to one side of the body and becomes incorporated into a gonad. Thus, the entoderm and germplasm arise from two common stem cells, but the remainder of the soma arises from other stem cells which have previously been separated from the undifferentiated line. Whether or not the daughter entoderm and germ cells, which arise from the same stem cell, go to the same side of the body is not apparent; all four cells are arranged in a medial line at one stage.

The identification and tracing of germ cells from the vegetal pole of the fertilized egg to the genital folds in anurans was reviewed by Di Berardino (1961). Her identification of the same cells in the mid-blastula and early gastrula stages of Rana pipiens indicated that the nuclei involved in nuclear transplants of the same species were not derived from germ cells. Thus germ cells can differentiate in frogs which develop from eggs containing somatic nuclear transplants.

In most species studied, the germ cells cannot be identified by their morphology or location until a number of them have differentiated. Sonnenblick (1950) reviewed the origin and migration of germ cells in Drosophila. Hathaway and Selman (1961) contributed more experimental evidence. From 2-11 cleavage nuclei migrate into the poleplasm where they

divide and form about 55 pole cells. Interblastodermal migration of some of the pole cells takes place prior to invagination; these cells contribute to the mid-gut epithelium. The remaining 30-40 pole cells enter the body through the posterior invagination; approximately half of them are incorporated into the gonads and half are apparently lost. In man, the germ cells can be identified in the endodermal yolk sac epithelium at the 12-13 somite stage, but already they number from 30-50 (Witschi, 1956). Likewise in the mouse, the germ cells number from 15-76 before they can be identified in the yolk sac splanchnopleure of the 8-day-old embryo (Mintz and Russell, 1957).

From the studies of mosaics in Drosophila, it was believed for a number of years that the germ cells arose from a single stem cell (Morgan and Bridges, 1919; Muller, 1920). Mosaics bred as though they possessed only one gentotype; the gonads were the same sex in gynandromorphs. However, when it was discovered that the gonads, and also the derivatives of separate imaginal discs, could be a mixture of male and female tissue in gynandromorphs, cleavage was considered to be indeterminate in Drosophila (Patterson and Stone, 1938). Cytological evidence that the early cleavage spindles were arranged at random with regard to the egg axis contributed to this conclusion. It appeared that more than one stem cell contributed to most cell lines including the germ cell line.

Even though the mixed types became numerous in the vast number of mosaics which were reported in Drosophila, the fact still remained that the majority of the mosaics were of the half and half types (Muller, 1954). Sonnenblick (1950, pp. 89-90) acknowledged the evidence which

indicated that indeterminant cleavage was the developmental basis for the varied gynandromorphic types, but he also indicated that the final patterns did not generally support this interpretation.

> However, the tendency in many, and perhaps the majority, of the mosaics is toward a bilateral arrangement in the distributional pattern of male and female parts. Since the position of the first cleavage spindle, indeterminate though it is, will directly influence the cleavage patterns which follow, analysis of sufficient material containing first cleavage spindles should indicate a preponderance of spindles arranged bilaterally, or nearly so, rather than in other possible positions.
>
> Despite the indeterminant nature of most of the early developmental processes, checkerboard patterns with complex distribution of parts are seldom found. The first cleavage spindle must have significant directive capacity in determining the position of the ensuing cleavage products. The cytoplasm may, furthermore, have sufficient rigidity to prevent indiscriminate drifting and nuclear movement; otherwise crazy-quilt distribution patterns ought to be more prevalent.

Re-examination of the sources of mixed gynandromorphs in most cases reveals that the genetic change probably occurred after several cleavages had taken place, or else more than one change took place in more than one cell line. Thus, mixed gonads and non-bilateral organization of somatic tissue could be expected.

Mixed types of mosaics were first produced in notable quantity in the experiments with the "claret" mutant in Drosophila simulans. Through the use of suitable markers, it was apparent that the maternal X chromosome was missing in some tissues of the gynandromorphs. Wald (1936) investigated the maturation and cleavage divisions cytologically. Because of faulty spindle formation, the X chromosome was frequently omitted from the female pronucleus but often included again in a daughter nucleus of

one of the subsequent cleavages. Thus, many of the zygotes started out as monosomics but regained the normal chromosome complement in part of their cells. The mosaic parts, of course, did not conform to any particular arrangement. If the eliminated chromosome were not incorporated into an early cleavage nucleus, gradual degeneration prevented its inclusion in nuclei which were formed later; if a gynandromorph were produced, the altered area had to be large.

The bulk of gynandromorphs have come from specific locus tests in which wild-type males are treated with radiation or mustard gas and then mated to females homozygous for specific recessive genes. Mutation, inactivation or loss of the wild-type allele allows for expression of the marker in a mosaic region. When sperm are treated with mustard gas, a genetic change may not be realized until cleavage or later mitoses in the embryo (Auerbach, 1945). The effect is usually immediate when sperm are treated with X-rays; however, through a sequence of events it is possible for only a fourth or less of the body to be altered in the mosaic progeny.

Bonnier et al. (1949) suggested a mechanism whereby loss of chromatin could occur in cleavage divisions after X-ray treatment of sperm. If a break in unsplit chromatids should occur in the sperm, one chromatid could restitute while the fragment of the other could become lost or translocated during the first cleavage of the egg. The broken ends of the sister chromatids, formed in the deficient daughter cell, would likely fuse. In subsequent divisions, a fusion-bridge-breakage cycle could occur in which additional material distal to the centromere would likely

be lost in each division until finally the entire chromosome would be eliminated. However, healing of the broken ends could halt the fragmentation process at any time and result in a deficiency which should be faithfully duplicated in subsequent cell divisions. If a recessive gene were located near the centromere, its presence might not be revealed in the somatic cells until several fusion-bridge-breakage cycles had uncovered it. If more than one marker were involved, the distally located genes might be revealed in one stable deficient type in one part of the body and all the markers revealed in another region. Bonnier et al. observed two gynandromorphs in which the male tissue was a mosaic of two genotypes, each expressing a different combination of sex-linked markers. The order in which the markers were expressed led to the suggestion that the chromosomes must have been looped at the time of breakage. Two breaks occurred in the chromatid: the distal part was lost; restitution of the part containing the markers took place in such a manner that the markers were inverted. The fact that very few mosaics exhibit two different mutant areas does not imply that the mechanism is infrequent in treated material. Very few markers are expressed over the entire body; hence, very few sectors reveal more than one of the mutant genes involved. In most cases the fusion-bridge-breakage cycle would eliminate the entire chromosome; the result usually would be no different than if the entire chromosome had been eliminated at one time.

Hinton (1959) believed that fusion-bridge-breakage cycles during cleavage were responsible for the ring chromosome loss and development of gynandromorphs in $\underline{w}^{vc}$ ring-rod chromosomal heterozygotes. Gynandromorphs

occurred frequently enough that he was able to study the process cytologically as well as genetically.

Although the occurrence of gynandromorphs with mixed gonads does not necessarily refute the hypothesis that all germ cells are derived from one stem cell, there are at least 6 cases of bilateral mosaics which indicate that an alternative condition is more likely. The soma and germ cells of each side possibly arise from two stem cells, one for each side of the body as shown in Fig. 9.

Mohr (1923) found a bilateral mosaic male which proved to carry a new allele of "singed", a sex-linked mutant which affects the bristles. Seven daughters of the mosaic carried the new allele, but 3 did not; hence, the germinal tissue must have been mosaic.

Panshin (1935) reported a bilateral mosaic male which exhibited two different alleles of "lozenge", a sex-linked mutant affecting eye color. The mosaic proved to be germinal; when mated to attached-X females, he produced the following sons: 18 expressed one lozenge allele, 3 the other lozenge allele, and 1 the wild-type allele.

Auerbach (1945) reported another mosaic male in which the eyes exhibited new alleles at the "white" locus. Bilateral expression was not perfect; each eye contained a small sector of the color shown by the other eye. Breeding tests revealed that the germinal tissue was mosaic for the same two alleles.

Mickey (1951) observed a mosaic male whose dam had received a cold shock treatment at an early embryonic age. The entire right eye showed a new "rough" phenotype, but the mosaic sired only 1 rough type in 788

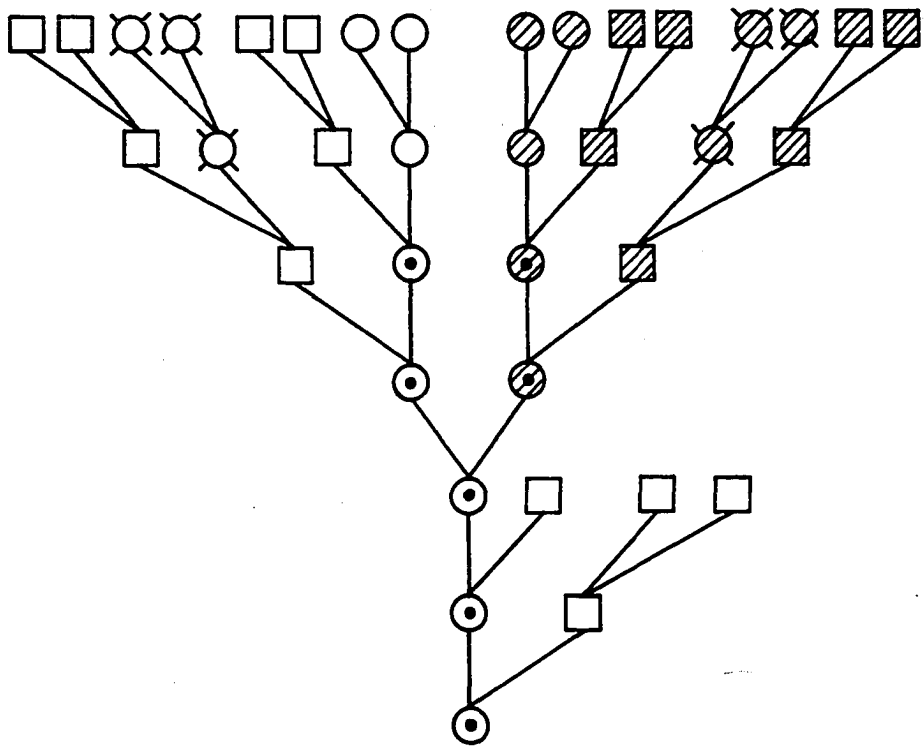


Fig. 9. Derivation of the germ cell line from two stem cells

This arrangement could account for mosaics that are bilateral with respect to both the gonads and the soma. A single genetic change, occurring after a given number of cleavage divisions, affects only half of any one tissue. Refer to Fig. 8 for legend.

offspring. The mutation behaved as an autosomal dominant in subsequent generations; segregation was normal.

Katz (1961) reported a male in which both the soma and germ cells were mosaic for a new white eye mutation. All of one side and part of the other was white in the eyes and testes sheaths. The mosaic produced 97 white eyed and 289 wild-type sons when bred to attached-X females.

Hollander (1962) found a bilateral mosaic male in which one eye was white and the other reddish. The parents were a white-eyed male and an attached-X female. Mosaicism was germinal; when bred to attached-X females, the mosaic produced 152 white and 15 reddish sons. The reddish characteristic behaved as an ordinary sex-linked recessive in subsequent crosses.

Panshin and Auerbach found their male mosaics in progeny from treated males and attached-X females; both decided that the mutations were probably induced in the paired chromatids of the sperm. Since Auerbach did not publish progeny test data and Mohr was only able to test 10 gametes, it is not possible to determine whether or not the gametic ratios deviated significantly from the 1:1 ratio expected from bilateral mosaics. However, the gametic ratios obtained by Panshin, Mickey, and Hollander can be considered as a significant departure.

Even if the germ cells in Drosophila are differentiated in two separate fields, one for each side, there are many factors which could unbalance the gametic ratios in bilateral mosaics. Any imbalance between sides in the (1) inclusion of nuclei in the poleplasm, (2) mitoses of

pole nuclei, (3) interblastodermal migration, (4) migration to the gonads, or (5) loss of pole cells, would greatly disturb the ratio, since the number of cells is small during all of these processes. The most deviate ratio, if repeatable with consistency in several mosaics would reflect the total number of germ cells which were actually incorporated into the gonads. In the case reported by Hollander for example, it appears that at least 11 pole cells must have migrated to the gonads in order for a 10:1 ratio to have been obtained. The ratio produced by Mickey's mosaic, no doubt, will prove to be an exception when data have been collected on many more bilateral mosaics. Some germ cells may migrate to an environment which is not as favorable for mitosis as other sites in the gonad. Unless each mosaic is tested at a constant rate over an extended period of time, stratification of the mosaic germinal tissue can also unbalance the gametic ratio (Whiting, 1961). Thus, a large number of mosaics would have to be adequately tested in order to determine whether or not the gametic ratio generally is 1:1 in bilateral mosaics. Cases were reviewed here to illustrate what information might be gained if more mosaics were progeny tested and reported.

Carlson and Southin (1963) found that the average mosaic gonad was 25-50 percent mutant in their experiments involving chemical mutagenesis. They concluded that the descendents of only 2-4 cleavage nuclei were incorporated into the gonads. Apparently the cleavage nuclei formed aggregates from which adjacent nuclei migrated to the polar cap. Thus, mosaicism of the germ cell population did not correspond to the degree of mosaicism in the embryonic pool of nuclei.

Even if the two halves of the body were formed from two stem cells in Drosophila, it would still be possible for some cell lines to differentiate from more than one stem cell on each side. The number of stem cells depends on the number of embryonic fields which provide sites for differentiation. Stern (1954a, 1954b, 1955) was able to determine some of the relationships between gene action and embryonic fields in his studies on Drosophila mosaics. For example, the differentiation of one bristle within an embryonic field in some way prevented like differentiation of any other cell within the same field. The bristle normally differentiated at the center or 'peak' of the field. But when the field was mosaic and the peak was occupied by cells which were genetically unable to respond, one and only one bristle was formed by the nearest cells which could respond. In the case of bristle formation there were many fields located in specific regions over the body. The differentiation of sex combs normally took place in two fields, one for each foreleg. If male tissue occupied the peak of the field, a sex comb developed in its normal position. If female tissue occupied that region in a gynandromorph, the sex comb appeared in the male tissue in closest proximity within the field. The number of fields was under gene control. In some mutants, fields for sex combs apparently existed on all three pairs of legs and also on the antennae when they were converted to legs.

For species in which bilateral contrasts of the soma and germplasm of mosaics are the exception rather than the rule, the germ cells may be descended from more than one stem cell in one field or else one or more stem cells in several fields. For example, if two or more cell lines

contain genetically changed cells but less than one-fourth of the tissue in one or more of the lines is affected, then each of those lines must have arisen from four or more stem cells (Fig. 10).

Bilateral mosaics are rare in the mouse. Even the gynandromorphs reported by Hollander et al. (1956) were asymmetrical. Russell and Major (1956) observed that "pearl" (pe) homozygotes are prone to a high frequency of reverse mutation which affects both the pigment and germ cell lines. Five such mosaics occurred in 360 animals; individual animals having 5, 50, 80, 100 and 100 percent of the coat expressing the wild-type allele produced respectively 18.2, 52.7, 43.6, 38.5 and 36.2 percent wild-type progeny. Dunn (1934) reported a "chinchilla" (c^{ch}) male heterozygote in which the wild-type allele had mutated to another allele in about 5 percent of the pigment cells and 21 percent of the diploid germinal tissue. In progeny tests, the mosaic produced a ratio of 20 gametes containing the new allele to 170 containing either c^{ch} or +. Since only 5 percent of the coat was affected in two of the reported mosaics, it appears that as many as 20 stem cells could contribute to the pigment cell population provided that an equal amount of coat were pigmented by the descendents of each one. In a strain of non-agouti (a) mice, Bhat (1949) found a female mosaic in which 6 percent of the diploid germinal tissue and an undetermined amount of the coat was heterozygous for the "white-bellied agouti" (A^W) allele. The mosaic produced 61 aa and only 2 A^W/a progeny when bred to non-agouti males. The somatic cells which carried the mutation probably did not include the pigment cells, since the effects at the non-agouti locus are determined primarily by the

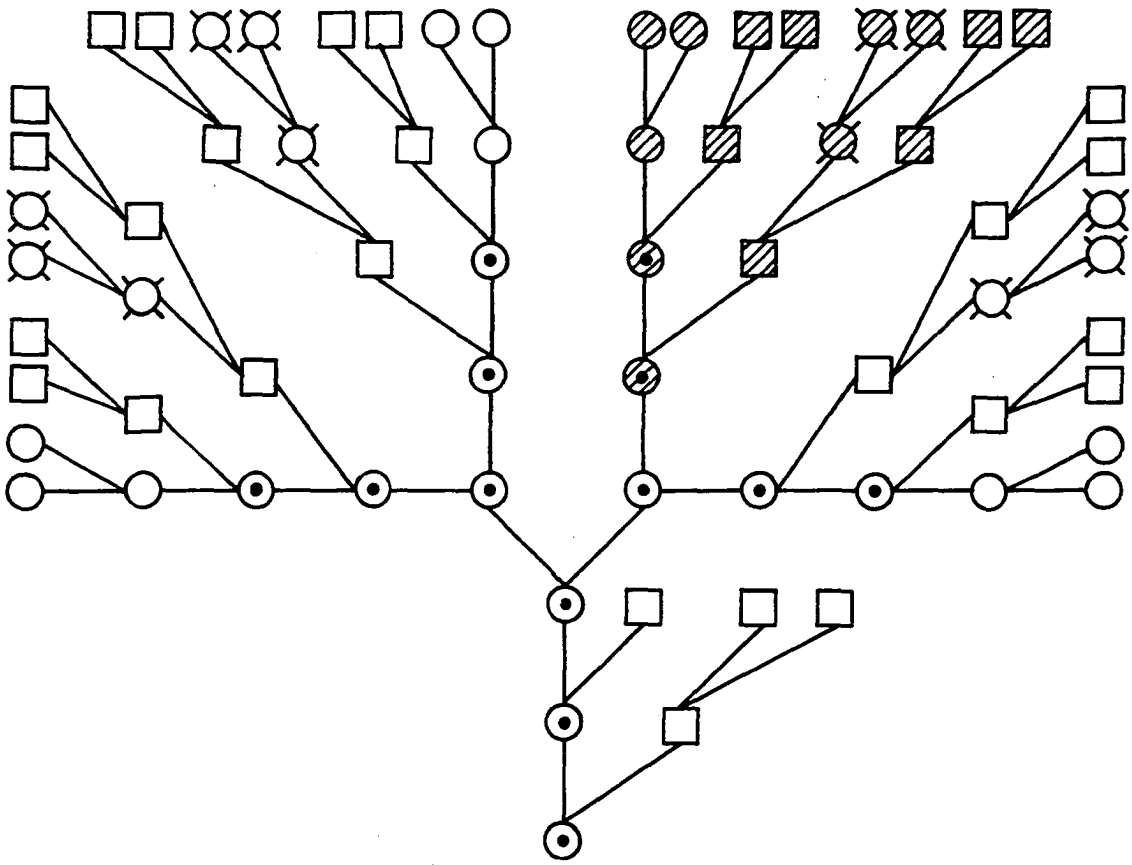


Fig. 10. Derivation of the germ cell line from four stem cells

This is a more generalized arrangement for species in which mosaics are not usually bilateral. The scheme could be extended to include species in which the germ cell line is derived from more than four stem cells. Note that continuity of the germline is still maintained. Refer to Fig. 8 for legend.

hair follicles rather than the melanocytes (Silvers, 1961). Whatever the cells are, their line apparently does not become separated from the germline until relatively late. If 61:2 is the most extreme gametic ratio that can be produced by a mouse which is mosaic for both the soma and germplasm and that ratio is repeatable with consistency, it appears that germline could be derived from as many as 16 stem cells.

When mosaics involving both germinal and mosaic tissue are found, an attempt should be made to determine the genetic composition of the germ cells in each gonad. This determination is particularly important when somatic changes are exhibited on only one side; the result may indicate whether or not both the germplasm and soma of each side differentiate in separate right and left fields.

In the transplantation studies reviewed by Tyler (1955), it was determined that the germ cells do not cross over from one side to the other during the development of salamanders. However, in most species of birds, germ cells do cross over from the right to the left side during their migration to the gonads (Witschi, 1961). Genetic support is given to this interpretation through breeding tests of a mosaic rooster which showed yellow skin (ww) on the left and wild-type skin (w/+) on the right side (Crew and Munro, 1938). Semen was collected from each testis separately by a technique of cloacal entry; Brown Leghorn (ww) hens were inseminated artificially. The 11 yellow and 9 wild-type progeny produced from the right testis were well within the expected 1:1 ratio. The fact that 6 wild-type as well as 11 yellow progeny were produced from the left testis indicates that germ cells did cross over from the right side.

A more critical case would involve a homozygous genotype on the right and a contrasting heterozygous, or preferably homozygous, genotype on the left. Then the right testis should produce only one kind of sperm and the left a mixture. Of primary importance is the technique of gamete sampling which is possible in mosaic roosters but apparently attempted in only this one case. Sampling gametes from each side in hens is not possible; only the left gonad is functional. It is doubtful that the differentiation of testes and ovaries in the chicken is dependent on the autonomous action of genetically different germ cells; all seven cases of gynandromorphs reviewed by Cock (1955) were female on the left and male on the right. This interpretation is further supported by experimental evidence that the right gonad will develop into a testis if the ovary of a female is removed at an early age.

By means of unilateral castration, Wright and Eaton (1926) made a belated attempt to determine the genetic composition of the germ cells in at least one testis of a mosaic guinea pig; unfortunately only one litter was sired after the operation. Previously the mosaic had sired 78 wild-type and 147 "dilute" ($\underline{c}^{d}$) progeny when bred to dilute females. The mosaic's parents were both $\underline{c}^{d}\underline{c}^{d}$; hence, a reverse mutation to wild-type must have been included in part of his pigment cells and germ cells. Because the one litter arriving after the operation contained one wild-type and two dilute young, Wright and Eaton concluded that the right testis produced both alleles. In female mammals the separate ovaries of mosaics could possibly be tested by means of ovary transplantation.

Because mosaicism, which affects both the soma and germplasm in the

same individual, has been reported in several species (chicken, Cock, 1955; pigeon, Hollander, 1949; guinea pig, Wright and Eaton, 1926; mouse, Dunn, 1934, Bhat, 1949, Russell and Major, 1956), it seems safe to conclude that all germ cells are not derived from one stem cell in the mammals and birds as they are in Ascaris and Miastor. Bilateral symmetry of mosaics in untreated material seems to be the rule in Drosophila and chickens; hence, it seems likely that both the germplasm and soma of each side arise from a single separate stem cell. The gametes derived from each side in Drosophila and chickens, and probably most other species, cannot be expected to completely coincide with the phenotypes of their respective sides, for the germ cells become mixed during migration. In mammals and many species of birds, bilateral symmetry of mosaics is the exception; the amount of genetically changed tissue of both the germplasm and soma is often far less than half. Thus the germ cell line and the somatic cell lines must arise from several embryonic fields, or else from several stem cells in one field, in the majority of mammals and birds.

Proliferation of cells in later stages

Mosaics in which presumably only one cell line is affected by a genetic change will be considered in this section. Mostly somatic mosaics will be discussed since mosaicism of the germline does not give rise to a phenotypic pattern. The gametic ratios involving independent loci would not be changed by germinal crossing-over or nondisjunction if the daughter cells were equally viable. Germinal crossing-over between linked markers could possibly be detected by a higher frequency of cross-

over gametes than expected.

Somatic mutation in two alleles at the "miniature" locus in Drosophila virilis was found to occur only after the separation of the germinal and somatic cell lines (Demerec, 1941). One allele was unstable in both the soma and germplasm; another mutated only in the soma. Demerec stressed the point that the dominant allele was always the stable one in all cases which he studied; hence, somatic mutation never proceeded from the dominant to the recessive. If the mutation occurred relatively early in ontogeny, a large spot expressing the dominant allele was formed; but, inside it there never was another spot indicating a change back to the recessive. If a somatic mutation to another unstable allele did occur, then mutation back to the original or still another allele did take place on occasions within the same spot. Frequencies of somatic mutations in some cases were dependent upon sex, age of dam, and genetic background. Four specific genes at other loci were found to increase the frequency of somatic mutation of both unstable alleles for miniature; another gene increased mutation of one of them only in the germline.

Lewis (1959) reported "ivory" (w^i) to be an unstable gene in Drosophila melanogaster. The frequencies of reversions to wild-type in both the germ and somatic cells was greatly increased by X-ray treatment of the female and early larval stages respectively.

Green (1962) concluded from X-ray experiments on the white alleles and mutants of other loci in Drosophila that the nature of back mutations was a characteristic of particular alleles rather than loci. For each

gene the back mutation rate and qualitative nature of the back mutant was distinctive.

Chase (1939) suggested that the tortoiseshell patterns of mammals likewise could be the result of unstable genes. When the factor producing tortoiseshell is present in a species, there is evidence that it is usually allelic to recessive yellow. The yellow mutation is found in most domesticated rodents and carnivores (Little, 1958), swine (Hetzer, 1945), and cattle (Ibsen, 1933). In the yellow mutants of some species, somatic mutations to an allele producing black pigment have occurred: guinea pigs (Ibsen, 1919; Chase, 1939), hamsters (Robinson, 1957, 1963), dogs (Little, 1957), cats (Bamber and Herdman, 1927), swine (Warwick, 1931) and cattle (Ibsen, 1933). For species in which the tortoiseshell pattern is determined by an allele of yellow, the effects of the alleles seem to differ primarily in that somatic mutations are much more numerous and occur earlier in ontogeny in tortoiseshell than in yellow mutants.

In the guinea pig, yellow ($\underline{e}$), tortoiseshell ($\underline{e}^{P}$), wild-type (+) and dominant black (E) form an autosomal multiple allelic series (Ibsen, 1919). From the phenotype of the tortoiseshell individuals, it appears that $\underline{e}^{P}$ is really an unstable form of $\underline{e}$ which mutates to + (Chase, 1939). The background color is yellow; the irregular spots and bands are made up of wild-type hairs. If the animal is homozygous for non-agouti, the spots contain solid black hairs.

A similar multiple allelic series exists in the rabbit: recessive yellow ($\underline{e}$), tortoiseshell (Japanese) ($\underline{e}^{J}$), wild-type (+), steel ($\underline{E}^{S}$), and dominant black ($\underline{E}^{D}$) (Robinson, 1958). Unfortunately, some of the

terms of rabbit fanciers are not consistent with those pertaining to other rodents; yellow, non-agouti rabbits are referred to as tortoise-shell and yellow rabbits with black spots as Japanese. In order to have consistency of terms among species, the phenotype produced by $\underline{e}^J$ will be referred to as tortoiseshell and $\underline{e}$ as yellow in this thesis. A black phenotype is produced by $\underline{E}^D$ in the homozygote and in compounds with other mutant alleles. The heterozygote ($\underline{E}^D/+$), however, is only dark agouti; the subterminal yellow band of the agouti pattern is not eliminated completely. In tortoiseshells ($\underline{e}^J$), it appears that somatic mutation proceeds from a state producing yellow phenotype ($\underline{e}$) to a state producing black phenotype ($\underline{E}^D$). The fact that the hairs within the mutated areas are solid black in the homozygote ($\underline{e}^J\underline{e}^J$) and compound ($\underline{e}^J/\underline{e}$) but dark agouti in the heterozygote ($\underline{e}^J/+$) (Punnett, 1924), indicates that mutation does proceed to $\underline{E}^D$. In the heterozygote the dark spots are much more distinct against the light-colored belly than against the agouti-colored back. In describing the effects of the alleles at the yellow locus, it is assumed, of course, that the remainder of the genotype affecting coloration is wild-type. In black and tan ($\underline{a}^t$) rabbits which are heterozygous for tortoiseshell ($\underline{e}^J/+$), black spotting can be observed on the tan belly.

Hetzer (1945) considered that yellow (including all shades through dark red), tortoiseshell, and black in swine were determined by a multiple allelic series similar to that of the guinea pig. The F_2 of the cross, Hampshire (dominant black) X Duroc-Jersey (yellow), contained 45 black and 21 yellow pigs (Lloyd-Jones and Evvard, 1919). The backcross

of the F_1 to the yellow parent of a Black Mulefoot X Duroc-Jersey cross produced 19 black and 23 yellow pigs (Detlefson and Carmichael, 1921). Backcrossing to the tortoiseshell parent of a Hampshire X Berkshire (tortoiseshell) cross, produced 7 black and 2 tortoiseshell progeny (Schmidt and Lauprecht, 1936). Crosses between tortoiseshell and yellow breeds showed that they differed in the factor for black spotting. The tortoiseshell pattern of the Berkshire was dominant to the self yellow of the Duroc-Jersey (Wentworth and Lush, 1923). The F_2 gave 111 tortoiseshell and 37 yellow; the backcross to the yellow parent produced 20 tortoiseshell and 15 yellow. A cross of Duroc-Jersey X Poland China (tortoiseshell) gave similar results (Warwick, 1931); the F_2 consisted of 161 tortoiseshell and 55 yellow progeny.

If the various shades of yellow are disregarded, most of the breeds referred to above have been shown to differ from wild-type by only one factor governing the distribution of black and yellow pigments. In a Tamworth (yellow) X wild-type cross, backcrossing to the yellow parent gave 5 wild-type and 3 yellow (Wentworth and Lush, 1923). Kosswig and Ossent (1932) obtained 29 tortoiseshell and 83 wild-type F_2 progeny from the cross of Berkshire X wild-type. The lack of solid yellow pigs in the F_2 showed that the yellow background color and black spotting of the tortoiseshell pattern could not become dissociated; that is, both characteristics were determined by the same factor. Schmidt and Lauprecht (1936) observed that self black was only incompletely dominant to wild-type; the F_1 progeny were black with brown stripes. They summarized the F_2 ratios which had appeared in the literature, 13 black: 3 wild-type.

Since each of the yellow, tortoiseshell, and black breeds differed from wild-type by only one factor governing the production of yellow and black pigments, reversion to wild-type could have been expected in the two-factor crosses given above if the factors involved were not alleles. No reversion to wild-type was indicated except in the F_2 and F_3 of the Berkshire X Duroc-Jersey cross (Wentworth and Lush, 1923). Then the longitudinal striping appeared as a temporary juvenile pattern on otherwise yellow pigs.

A more satisfactory test for allelism would involve three alleles. Crossing both tortoiseshell and yellow to wild-type and then mating an F_1 progeny from each cross together should produce a ratio of 3 wild-type: 1 tortoiseshell if tortoiseshell and yellow are allelic or all wild-type if they are not allelic. Interaction between non-alleles in the double heterozygote is a possible, but unlikely, drawback to the test. A test involving only domestic breeds would be (self black X tortoiseshell) X yellow. If all three factors are allelic, only black and tortoiseshell progenies could be expected; that is, no wild-type could be produced. No doubt this last test of allelism has been made, but not recorded, many times in rotational crossbreeding programs by the following matings: (Hampshire X Berkshire or Poland China) X Duroc-Jersey or Tamworth. This test, like the breed crosses referred to in the literature, is only good if the breeds are free of other mutants which could be confused with the presumed alleles. Genes producing non-agouti (recessive self black) and self yellow are known to be produced by the non-agouti locus in some other mammals (Little, 1958).

The effects of the tortoiseshell allele are more nearly alike in swine and rabbits than in swine and guinea pigs. The phenotypes obtained by Punnett (1924) in crosses of tortoiseshell and wild rabbits are very similar to those obtained in crosses of Berkshire and wild swine. In both cases the F_1 progenies were wild-type except for black spots which were particularly apparent on the light-colored belly of the wild-type pattern. Schmidt and Lauprecht (1936) included excellent photographs of such pigs obtained in repeated backcrosses of the wild-type allele to the Berkshire breed. The tortoiseshell allele in swine and rabbits apparently is an unstable form of yellow which mutates in the pigment cells to the dominant black allele rather than to the wild-type allele as in the guinea pig.

Wentworth and Lush (1923) observed that the background color of the F_2 tortoiseshell segregates in crosses of Berkshire X Duroc-Jersey was predominantly light yellow as in the Berkshire parent. They concluded that the loci determining the black spots of Berkshires and the darkening of yellow to red in Duroc-Jerseys must be linked. They also noted that red spots occurred on the light yellow backgrounds of tortoiseshell segregates, as in the Berkshire breed, but not on the light yellow segregates which had no black spots. Possibly, factors similar to those found in maize (McClintock, 1956) are suppressing dominant black to the recessive yellow state and a darkening factor to a dilute condition. Release of the suppressing elements in the pigment cell lines would result in black spots and red spots on a light yellow background. The transposition of a suppressing element to the darkening factor could

possibly account for the two F_1 offspring having dilute yellow background color instead of red in the Duroc-Jersey X Berkshire crosses reported by Severson (1917).

Hetzer (1954) observed that the size of spots on tortoiseshell segregates recovered from Landrace X Poland China crosses was much reduced in comparison to the tortoiseshell ancestor. He was able to regain the parental type by selection and concluded that the Poland China breed had modifiers which extended the size of spots. In comparison, Demerec (1941) found a gene in Drosophila virilus that induced an unstable gene for miniature to mutate at an earlier stage; the size of the altered areas was larger in the presence of the modifying gene. McClintock (1956) observed that somatic mutations occurred later when the dosage of the controlling elements of maize was increased. In fact, mosaic spotting was nearly eliminated when the dose was high. It may be that Duroc-Jerseys and Tamworths carry either so high a dosage of controlling elements that no spots appear or else no controlling element at all so that the yellow is stable. Thus, when they are crossed to breeds having the opposite concentration of controlling elements, the yellow descendents could conceivably be spotted. Even wild-type swine could be a source of controlling elements; McClintock has found controlling elements to be widespread in maize. Wentworth and Lush (1923) observed some yellow pigs with small spots in the F_2 and backcross to the yellow parent of the Tamworth X wild-type cross. It is surprising, however, that there were no black spots on the F_1 offspring, not even on the bellies. In the F_2 tortoiseshell segregates of the Duroc-Jersey X

Berkshire cross, the black spots were much larger on pigs having light rather than dark background color. Likewise, the exceptional F_1 pigs which exhibited light background color had much larger black spots than any of the other F_1 progeny which Severson (1917) observed in a cross of the same breeds. Wentworth and Lush considered Wright's interpretation; there may be a physiological reason that red swine do not have large black spots. The fact, that heterozygotes of the tortoiseshell and wild-type alleles exhibit black spots on the belly but not on the back (Schmidt and Lauprecht, 1936), indicates that black mosaic spotting and dorsal striping are also physiologically incompatible.

If transposition of a suppressing element or mutation of a more conventional nature is responsible for the black spots and red spots on a yellow background in mammals, most of the changes must be somatic; the writer has found no report of germinal changes. Self black offspring have been produced by tortoiseshell parents in guinea pigs (Ibsen, 1919; Chase, 1939) and both self black and self yellow in rabbits (Robinson, 1958), but the exceptional types have always bred as tortoiseshell when tested. When the exceptional F_1 sow reported by Severson (1917) was backcrossed to a Berkshire boar, she produced 8 offspring like herself and mate (light-colored background) and 1 offspring with red background color. The genetic change, which resulted in the sow having light background color, could be interpreted as a germinal as well as somatic mutation especially if the one pig with red background color were misclassified. The misfit offspring was also an exception to the hypothesis that red pigs cannot have large black spots. Hence, it may

have been basically a light yellow in which the few non-black areas had mutated back to red; 2 of the 8 sibs having light background color also exhibited red spots.

The finding of an occasional tortoiseshell F_1 progeny in crosses of dominant black breeds X tortoiseshell breeds of swine, as well as additional F_1 offspring showing light background color in crosses of tortoiseshell breeds and red breeds, would greatly strengthen the possibility that suppressing elements are causing mutability in swine. In a similar manner it should be possible to produce tortoiseshell guinea pigs in which somatic mutation proceeds to $\underline{E}$ instead of +. Compound types ($\underline{E}/\underline{e}^P$) would be tested for the new allele by crossing to $\underline{ee}$. Tortoiseshell rabbits with wild-type instead of black spots might be produced by crossing $\underline{e}^J/+$ X $\underline{ee}$. That such a change has not been reported, does not mean that it could not happen. Transposition of the suppressing element to a specific dominant gene is a rare event in maize. Such a change was expressed at the A_1 locus in only one kernel in 71 ears and at the A_2 locus in one kernel in each of 3 out of 120 ears in McClintock's (1953) experiments. The change would be even more rare in animals than in maize since the germinal and somatic cell lines separate earlier in ontogeny. The $\underline{e}$ locus is the only one which has been reported to be labile to variegation in guinea pigs, rabbits, and swine. Because of the emphasis on inbreeding in guinea pigs and pure-breeding in rabbits and swine, the number of heterozygotes in which transposition of suppressing elements could take place between alleles is rather small. Proof that the original tortoiseshell mutants arose from an $\underline{e}^+$ guinea pig,

an $\underline{E}^{D}$ rabbit and a dominant black hog would indicate that suppressing elements exist in mammals, but it is very doubtful that the origin of existing tortoiseshell types could ever be traced. Now that a dominant black has been reported in the mouse (Bateman, 1961), the stage is set for both types of tortoiseshell to appear by the action of a suppressing element.

Reverse mutation to wild-type seems to be the most likely cause of mosaicism in blue (Bl) Andalusian chickens. In heterozygotes, black areas occurring on a dilute-colored background indicate reversion of the partially dominant allele to wild-type. In homozygotes, dilution is more extreme; the background color is nearly white. Blue spots appear on the white background as the result of reverse mutation of one allele. Black spots occurring within the blue ones, as noted by Hollander and Cole (1940), indicate reverse mutation of the other mutant allele.

Hollander (1944) reviewed mosaicism including variegation in pigeons. The rare appearance of wild-type areas on the yellow type (recessive red, e) may be a condition homologous to that of mammals. Two closely linked loci on the sex chromosome control flecking patterns that occur consistently in certain mutants. Ash-red ($\underline{B}^{A}$), brown (b), and wild-type form an allelic series at one of the loci; almond (St), faded ($\underline{St}^{F}$), and wild-type at the other. The change responsible for flecking apparently can occur at any stage of growth; the amount of flecking, particularly in almond mutants, increases at every molt. The shade of the flecking in homozygous faded birds gives no clue as to whether the gene is reverting to wild-type, being inactivated, or being lost; faded is the

same intermediate shade in the hemizygote as in the heterozygote. No flecks are apparent in the homozygotes of $\underline{B}^{A}$ or $\underline{St}$ since both alleles operationally are fully dominant. If inactivation or loss is involved, the lack of gene action at the $\underline{b}$ and $\underline{St}$ loci results in brown and wild phenotypes respectively; ash-red females (hemizygous) show brown flecks, but almond females show wild-type flecks. The occurrence of both wild-type and brown flecks in $\underline{St}$ +/+ $\underline{b}$ males could be interpreted as the inactivation or loss of $\underline{St}$ alone in some melanocytes and both $\underline{St}$ and $\underline{b}^{+}$ in others. The fact that the flecks on $\underline{B}^{A}$ +/+ $\underline{d}$ birds were wild-type rather than dilute ($\underline{d}$), which is loosely linked, indicated that the entire chromosome was not lost (Hollander and Cole, 1940). Germinal changes to $\underline{St}^{F}$ and appearance of faded flecks in almond birds indicated that some form of mutation was occurring. Hollander suggested that flecking in the pigeon might be produced in a manner similar to the variegated position effects in Drosophila.

In a review of position effects, Lewis (1950) discussed the "spreading effect" of heterochromatin. When closely linked wild-type genes became associated with heterochromatin, usually by translocation or inversion, the heterochromatization process is believed to spread through them and suppress their action in some somatic cells. The affected individual may express variegation for several of the loci; a mutant effect of the locus located nearest the source of heterochromatin is expressed more frequently than effects of those located farther away.

Lewis considered the variegation of mottled white 258-18 in Drosophila melanogaster (Demerec and Slizynska, 1937) to be a good example

of the spreading effect. A reciprocal translocation between the X and fourth chromosomes placed the wild-type alleles of white and roughest next to the chromocenter of the fourth, the roughest locus being the closer to the source of heterochromatin. The translocation homozygotes were selected to form lines having either light or dark-colored eyes. Wild-type, rough, and rough-cherry spots occurred against a rough-cream background or else rough-cream, rough-cherry and rough spots against a wild-type background; that is, no mutant condition for color was expressed without roughest also being expressed. In one case a new line was formed from a single exceptional male in which variegation was the same except that the lowest mutant expression was white instead of cream. Because wild-type areas could occur on a white background and vice versa, Demerec and Slizynska believed that mutation was occurring in opposite directions in the two types. Cream and cherry appeared to be intermediate alleles to which mutation could also proceed.

Schultz (1941) considered the red spots on a white background to be groups of residual wild-type cells that had not changed genetically during development. Judd (1955) demonstrated that the wild-type allele of white, which he extracted from the translocated region by obtaining crossover chromosomes, had retained its wild-type properties. Thus, it could be assumed that the rearranged wild-type allele is not genetically changed in the germ cells. If so, it follows that the changes would be unidirectional (to the mutant state) and occur only in the soma. The fact that the ratio of wild-type to mutant cells can be altered to form a series varying from nearly wild-type to nearly all mutant type,

indicates that the same unidirectional change takes place in the development of both extreme types. Several factors in addition to selection can vary the ratio. Gowen and Gay (1934) observed that addition of an extra Y-chromosome decreased the number of white ommatidia. Culturing flies at high or low temperatures respectively decreased and increased the number of mutant cells. Schultz (1956) found that reciprocal changing of newly hatched larvae between cold and warm temperatures did not alter the pattern determined by the incubation temperature. He concluded that the change from wild to mutant type was irreversible and not a temporary suppression of gene activity.

It is surprising that extremes of temperature could influence variegation only during incubation. Gowen (1929) and Patterson (1929) demonstrated by X-ray experiments that cell division in the developing eye continues for two or three days after hatching of the larvae. Treatment of egg and larval stages resulted in mosaicism for heterozygous color markers. Most of the mosaics were induced at some period between one and three days of larval growth. X-raying of earlier stages produced large but infrequent mutant areas. A single mutant ommatidium in an otherwise wild-type eye often resulted from treatment at later stages.

Baker (1963) considered variegation in Drosophila as resulting from an alteration in the translation of the genetic message rather than a mutation of the gene itself. He compared the process leading to the development of pigment in variegated eyes to that of cellular differentiation. The same genes are present in all the cells but only operate in those which differentiate. The differentiated state is inherited in the

descendent somatic cells. For those cases of variegation in which no germinal mutations are ever found in extensive breeding tests, it remains a moot question as to whether or not "an inherited alteration in gene action" (Baker, 1963, p. 66) should be classified as different from gene mutation.

McClintock's (1958) experiments with maize have a bearing on the direction of mutation and the timing of control in variegated mutants. She described fully pigmented areas on otherwise colorless kernels. Within the large pigmented areas were again colorless regions which in turn showed specks of pigment. According to her interpretation, the colorless regions were formed by cells which divided during the time that the suppressing element was active and the dark areas during periods of inactivity. Thus, the gene alternated between active and inactive states; each state lasted for a period of several cell generations.

Assuming that mosaic spotting patterns of mammals and birds are formed by the migration of pigment cells out from centers in the skin, a trail of residual dominant type cells should pigment some of the hairs along the path between the pigment center and a ventrolateral spot if a residual genotype is maintained by some of the melanocytes. But if the spots are small and dispersed widely over the body, another interpretation seems justified; the melanocytes of each spot are proliferated from a pigment cell that mutates from the recessive condition at the site. Once the pigment centers and migration paths have been established in the various species in which variegated mutants exist, examination of the patterns should aid in determining whether the gene involved is in

the recessive or dominant state in the early embryo. If the locations of the pigment centers in swine are similar to those of most mammals, it appears that each of the small widely dispersed black spots, typical of spotted Poland Chinas or hybrids of Berkshires and Duroc-Jerseys, represents a mutation from yellow to black at the site of the spot. The effect will have to be demonstrated in more than one mutant and more than one species before conclusions can be drawn.

Cock (1953) attributed the flecking which involved the sex-linked dominant genes, barred (B) and silver (S), in the chicken to the loss of the terminal fragment distal to the centromere. He concluded that B was located nearer the end of the chromosome than S since flecking involved the loss of barred more frequently than silver. In coupling heterozygotes (B S/+ +), he found 257 silver, 87 wild-type, and only 1 barred flecks. Females appeared to lose B but not S. Cock suggested that a deficiency large enough to include S would probably be lethal to a melanocyte in the female (hemizygous). Homozygous barred males are paler in color than heterozygotes or barred females. Since no pale barred flecks appeared in B/+ males, somatic crossing-over was eliminated as a mechanism. In his interpretation, Cock failed to consider that a variegated position effect might be involved. Loss of a terminal fragment distal to the centromere during somatogenesis was believed for a number of years to be the cause of the yellow variegation associated with a long X-chromosome inversion in the scute-8 apricot stock of Drosophila melanogaster (Patterson, 1933; Noujdin, 1935). Later the phenomenon was considered to be a position effect (Lewis, 1950). If

flecking in the chicken should also be a position effect, B would likely be located nearer the source of heterochromatin than S, but not necessarily terminal and distal to the centromere.

A factor similar to "Dissociation" in maize (McClintock, 1956) could account for the variegation phenomena of pigeons and chickens. Dissociation allows for two mechanisms, reverse mutation due to transposition of a suppressing element and loss of an acentric fragment due to a break at the position of the suppressing element. All suppressing elements studied by McClintock produced recessive mutants by suppression of dominant alleles. However, there seems to be no reason to exclude dominant mutations from arising in the same manner. Dissociation is dominant itself in that it can cause chromosome breakage when present in only one dose.

Eight cases of variegation resulting from reciprocal translocations between the X chromosome and an autosome carrying either the wild-type allele of brown (b), pink-eyed dilution (p), or chinchilla (c^{ch}) have been investigated in the mouse (Russell, 1961, 1963; Russell and Bangham, 1961; Cattanach, 1961). The translocation heterozygotes were variegated if the intact autosome carried the recessive mutant allele and two X chromosomes were involved. Thus, XX females and XXY males were variegated; XO females and XY males were not. As in variegated Drosophila, the rearranged wild-type gene was reclaimed in its original form in crossover chromosomes.

The basis for the expression of variegation involving sex chromosomes in mammals was found by Lyon (1961, 1962) in the cytological observations

reported by Ohno and Hauschka (1960). In the female mouse, one X chromosome of diploid cells and two X chromosomes of the tetraploid liver cells are heteropyknotic. In the male the single X chromosome of diploid cells is not heteropyknotic; only one X chromosome exhibits heteropyknosis in the tetraploid nuclei. Since XO females arephenotypically nearly normal, Lyon reasoned that only the genes of one X homologue are required for normal development. To account for regions of wild-type and mutant effects in females, which were heterozygous for sex-lined genes such as dappled ($\underline{Mo}^{dp}$) and tabby ($\underline{Ta}$), Lyon postulated that the genes of the heteropyknotic X chromosome were inactivated and that the heteropyknotic state was not always assumed by the same X chromosome in all cell lines. Regions populated by cells in which the wild-type allele was inactivated would express a mutant phenotype. Areas in which the mutant allele was inactivated would be wild-type. A similar interpretation of sex-linked genes in man was given by Bentler et al. (1962).

Lyon (1963) presented additional evidence of the inactivation phenomenon, which she obtained by combining $\underline{Ta}$ and $\underline{Mo}^{dp}$ in the same animals but on opposite X chromosomes. The entire coat was a mosaic of mutant regions which expressed either the effect of $\underline{Ta}$ (lack of zigzag hairs) or $\underline{Mo}^{dp}$ (wavy hairs). Not all mutant hairs of the $\underline{Mo}^{dp}$ variegated type expressed the same anomaly: some were structurally abnormal only; some were white only; others were both white and structurally abnormal (Lyon, Mary F., Harwell, Didcot, Berkshire, England. Private communication. 1962). Thus, it appeared that the pigment cells autonomously expressed the white effect while certain other cells

of the hair follicle determined the structural effect. If separate cell lines of different origin were involved, the phase of gene expression (wild-type or mutant) would not likely be the same in both cell types of each hair follicle. By appropriate matings Lyon obtained female mice in which R($\underline{p}^+$), a rearrangement obtained from Cattanach (1961), was attached to the X chromosome opposite the homologue containing $\underline{Mo}^{dp}$; the autosomal component of the p locus was pp. (The notation "R($\underline{p}^+$)" is customarily used to represent the rearrangement of a locus, in this case the translocation of the wild-type allele of p to the X chromosome.) No areas expressing p only were found. Apparently, inactivation of the X chromosome containing R($\underline{p}^+$) and the wild-type allele of $\underline{Mo}^{dp}$ always resulted in the expression of $\underline{Mo}^{dp}$ and p in the same pigment cells. Cattanach (1963) observed "brindled" ($\underline{Mo}^{br}$) regions as well as regions of albino (c) or wild-type fur in mice which carried $\underline{Mo}^{br}$ on one X-chromosome and the wild-type allele of albino ($\underline{c}^+$) translocated to the other X. Since $\underline{Mo}^{dp}$ and $\underline{Mo}^{br}$ are not known to act autonomously in the melanocytes as do p and c (Silvers, 1961), neither Lyon's nor Cattanach's observations on double heterozygotes provide good reason for supporting or refuting the hypothesis that one or the other of the X-chromosomes is inactive in all somatic cells of the adult.

Lyon (1963) also obtained double heterozygotes of Ta and "striated" (Str) which provided much better test material. Abnormal hair morphology is an effect of each of the mutants. Neither mutant is the result of a rearrangement which requires the spreading of heterochromatin into an attached autosomal fragment in order to produce an effect. Lyon's

observations that both genes acted in the same regions in the cis type but in different regions in the trans type seem to be valid support of her hypothesis.

In a review of the genetics of mammalian sex chromosomes, Russell (1961) emphasized that the expression of heterochromatic effects requires at least two X-chromosomes, XX in females and XXY in males. Examples given were: (1) variegated position effects in the mouse, (2) sex chromatin in man and mouse, and (3) "brittle bristle" in hamsters. Brittle bristle apparently is only expressed when the short arm of the X becomes heterochromatic. Since the X is euchromatic in the male soma, the male can carry the trait but not express it. The heterochromatic state of one X in the female soma allows for expression of the trait. At meiosis the heterochromatic state of the X chromosomes changes in both sexes (Yerganian, 1961). Both X chromosomes become euchromatic in the female. The single X of the male becomes heterochromatic.

If it should be a general phenomenon in mammals that the X chromosome is heterochromatic when transmitted by the sire, then there might be an effect on the background color of variegated sex-linked mutants and rearrangements. That is, if the wild-type allele were transmitted through the sire and the mutant allele through the dam, the initial color would likely be mutant type; reversions would result in wild-type spots on a mutant background. Unfortunately, most of the sex-linked factors for color variegation in the mouse, the Mo alleles (Lyon, 1960) and rearrangements (Russell and Bangham, 1961) respectively effect lethality and sterility in the male. Some males heterozygous for a $\underline{c}^{+}\ \underline{p}^{+}$ rearrangement

with the X-chromosome proved to be fertile (Cattanach, 1961), but no data on the relative amounts of mutant tissue in the progeny were given.

One sex-linked mutant, tabby (Ta) which affects hair structure, can be transmitted by either parent. Kindred (1961) reported a maternal effect on the number of vibrissae in Ta/+ females. In six different strains, daughters of Ta sires and wild-type dams had fewer vibrissae (more mutant effect) than Ta/+ daughters of reciprocal matings. However, when tabby heterozygotes were repeatedly backcrossed to the C57 strain, Ta/+ daughters of Ta/+ dams had fewer vibrissae than Ta/+ daughters of wild-type dams. Only the effect obtained in the backcrosses to C57 would be expected if the action of the allele at the Ta locus were always suppressed by transmission through the sire. Kindred concluded that the genetic background determined the direction of the maternal effects. A special experiment, in which the sire was always the source of the Ta gene, was designed to show that the differences observed were maternal. When both Ta/+ and wild-type females were mated to the same Ta male sib, the Ta/+ daughters of the wild-type dams had significantly fewer vibrissae than the Ta/+ daughters of Ta/+ dams. In another experiment TaTa and Ta/+ females were mated to wild-type males; there was no difference between the groups of Ta/+ daughters (or Ta sons) from the two types of dams. Examination of Ta/+ daughters from crosses of Ta and + males with Ta/+ females would have allowed for a comparison of transmission through the sire with transmission through the dam; the maternal genotype would have been constant. Unfortunately such crosses were not reported.

The parental effects which Noujdin (1935, 1945) found in the scute-8

(sc^8), apricot (w^a) stock were confirmed cytologically by Prokofyeva-Belgovskaya (1947) in studies of salivary chromosomes. The X chromosome contained a long inversion which placed heterochromatin, normally associated with the centromere, next to the terminally located y ac loci. Some of the flies which were homozygous for the rearrangement showed mosaicism for what appeared to be yellow (y) and achaete (ac) even though only wild-type alleles were involved prior to the rearrangement; females were more frequently mosaic than males. The region near the distally located break, including the y ac loci, was heterochromatic 38.5 percent of the time in females and 16 percent in males. In the female, frequently the y ac region of one homologue was heterochromatic and the other euchromatic. When sc^8 w^a males were crossed to y ac v females, 246 of 1122 (21.9 percent) of the female offspring were mosaic for y ac as compared to 88 of 2022 (4.4 percent) in the reciprocal cross. Cytologically the sc^8 w^a chromosome exhibited the heterochromatic state in the y ac region 71 percent of the time when the derivation was paternal but only 20 percent when maternal. Just as in the hamster (Yerganian, 1961), the X was euchromatic in the sire, but the y ac region was transmitted in the heterochromatic state. The y ac region of only one X tended to be heterochromatic in the dam, but both X chromosomes were transmitted in the euchromatic condition.

Schneider (1962) reviewed the more recent work on the parental effects involving rearrangements of the wild-type allele of sex-linked white (w) in Drosophila melanogaster and presented data involving rearrangements of the wild-type allele of peach (pe) in Drosophila virilis.

Even though pe is autosomal and ordinarily located in heterochromatin, the results are very similar for both loci in both species. By the use of paper chromatography the amount of pigment in the testis sheath of males and in the eyes of both sexes was measured in the R(pe^+) heterozygous offspring of the various possible parental matings. In the crosses described, pe occupied the normal position of the locus. When R(pe^+) is transmitted by a heterozygous parent, more mutant tissue is present in the variegated offspring than when transmission is from a homozygous R(pe^+) parent. More mutant tissue is present in the offspring when transmission of R(pe^+) is through the sire than through the dam, regardless of whether homozygous or heterozygous parents are being considered.

The tortoiseshell pattern of female cats, which results from the heterozygous state of the sex-linked yellow mutation, was considered to be a typical variegated type by Lyon (1961). Her interpretation subsequently was strengthened by the cytological observations of Thuline and Norby (1961). They reported two sterile tom cats which had the chromosome constitution as determined by the sex chromatin pattern and a count of 39 instead of the normal 38 chromosomes in the tissue culture of the white blood cells. One of these cats was a tortoiseshell male; hence, XXY as well as XX can be variegated, while XY is not, in both the cat and the mouse. Since yellow is autosomal in all other mammals (Little, 1958), it is possible that sex-linkage of yellow may have evolved in the cat through a rearrangement becoming homozygous (Robinson, 1963). Dyte (1962) reported both sex-linked and autosomal inheritance of yellow in the Abyssinian breed of cats. He concluded that two different genes must be

involved, but he gave no data indicating that non-yellow progeny were ever produced from any matings of two yellow cats. It would be interesting to know if semisterility, which is indicative of translocations, occurs within the breed.

Robinson (1959) reviewed the literature regarding the occurrence of exceptional tortoiseshell males and exceptional non-yellow daughters of yellow males. None of the theories presented could account for all of the exceptions; application of the theory of variegated position effect to the data seems warranted at this time.

The fertile tortoiseshell reported by Bamber and Herdman (1932) produced: 8 yellow females and 10 yellow males (including one with minute non-yellow spots) from yellow mates; 13 non-yellow males, 14 tortoiseshell females, and 1 non-yellow female from non-yellow mates; 1 yellow male, 1 non-yellow male, 2 tortoiseshell females, and 6 yellow females (including one with minute non-yellow spots) from tortoiseshell mates. Bamber and Herdman suggested that a small fragment containing the wild-type allele of yellow from one X chromosome might have become attached to the intact X by "partial non-disjunction" in the tortoiseshell dam of the tortoiseshell male. They reasoned that the fragment must have become lost from most of the X chromosomes during spermatogenesis in the fertile tortoiseshell male.

Bamber and Herdman's explanation is essentially the same as what could be expected if tortoiseshell males are XXY and arise through nondisjunction in one parent. Cooper (1956) demonstrated genetically that male Drosophila which are XYYY in the soma can become fertile through

the "scaling down" (loss) of one of the extra Y chromosomes in the germ cells. Ford et al. (1959) reported an XXY-XX human mosaic in which the XX tissue was believed to have been scaled down from a potential XXY Kleinfelter trisomic. Because Ishihara (1956) made chromosome counts only from tortoiseshell male cats in which spermatogenesis appeared histologically normal, it is possible that the soma may have been XXY and contained 39 chromosomes even though only 38 chromosomes were found in the spermatogonia.

Theoretically, it would be possible for the XXY condition to be scaled down to an XY pair containing a different allele in separate cell lines within the same animal. Or, it may be that presence of some diploid tissue in the gonads or pituitary would allow for gametogenesis in the trisomic tissue. In either event, an equal number of gametes containing each allele would not be produced. Only 3 of the 56 gametes transmitted by Bamber and Herdman's tortoiseshell male carried the wild-type allele, even if the 2 yellow offspring with minute non-yellow spots are included in the class with the non-yellow daughter. Secondary nondisjunction may also have occurred in the fertile tortoiseshell male. A yellow male, one of 10 sons that were allowed to grow up, was sterile and exhibited behavioral and histological characteristics that are typical of sterile tortoiseshell males.

It may be that some of the yellow sires of exceptional non-yellow daughters were initially heterozygous XXY males that became fertile. According to Bamber's (1927) review, 9 of the 18 exceptional non-yellow females included in Doncaster's compilation were later reported to have

been sired by one particular yellow male (Bonhote, 1915).

Breeding records of only three other fertile tortoiseshell males are available, but those accounts are sketchy. One tortoiseshell male owned by a cat fancier was reported to have sired 3 yellow males, 1 yellow female, and 1 tortoiseshell female when bred to yellow females (Anonymous, 1956). Komai and Ishihara (1956) reported a tortoiseshell male which sired 1 tortoiseshell male, 1 tortoiseshell female, and 2 yellow females when bred to a tortoiseshell female. Jude and Searle (1957) reported a tortoiseshell male which had sired all three colors in both sexes including 11 tortoiseshell males. From their account it appeared that both yellow and wild-type alleles were being transmitted in the gametes.

Though specific matings should be made to test for parental effect on variegation in cats, there is some evidence in the literature which is applicable. If the X chromosome contributed by the sire were heterochromatic while that of the dam were euchromatic, as discussed above, tortoiseshells which received the wild-type allele from the sire should begin development with an effective genotype for yellow; those which received the yellow gene from the sire should initially be non-yellow. If alternation of the heterochromatization of the sex chromosomes took place in the melanocytes, the heterozygote, which was initially either yellow or non-yellow, would develop spots of the contrasting color. Failure of the alternation to take place would result in an anomalous yellow or non-yellow individual which would breed as a heterozygote. Four anomolous females were discussed by Whiting (1918, 1919): two yellow

females which were determined by breeding tests to be heterozygous and two females which were sired by a yellow male but were phenotypically non-yellow except for a few yellow hairs on one and small yellow spots on the other. Whiting believed that most exceptions to regular sex-linkage were overlaps. His interpretation was criticized on the grounds that, if modifiers were causing the heterozygotes to vary either to the yellow or non-yellow extreme, the same tendency should show up in the offspring (Bamber, 1927). Actually, the tortoiseshell kittens of the anomalous yellow females tended to vary more to non-yellow than to yellow. If parental effect on the heterochromatization of the X chromosomes were involved, the effects could only be expected to last for one generation. Hence, overlap of phenotypes could still account for all of the exceptional types (yellow females, non-yellow females and yellow males postulated to be XXY heterozygotes). The two nearly non-yellow heterozygous females and the dark tortoiseshell daughters of the anomalous yellow females, all received the wild-type allele from their dams; by the theory of parental effect on heterochromatization, they could be expected to approach the non-yellow color. If the anomalous yellow male reported by Bonhote (1915) were XXY as a result of parental nondisjunction, he would have received the wild-type allele from his sire and would be expected to be nearly yellow in phenotype. The dam of the anomalous yellow male was tortoiseshell. However, it is unlikely that maternal nondisjunction was involved; Russell (1961) stated that there was evidence for nondisjunction in male but not in female mammals.

If nondisjunction gives rise to XXY tortoiseshell males, it might be

assumed that the exceptional non-yellow females from yellow sires are the XO type (Frota-Pessoa, 1962). Such an assumption is not valid, since no exceptional yellow daughters of non-yellow males have been reported in what would be a comparable class. One of the exceptional non-yellow females reported by Bonhote was bred back to her anomalous yellow sire and produced 1 non-yellow male, 2 tortoiseshell females, and 1 of the exceptional non-yellow females. In producing those few offspring, the exceptional non-yellow female was shown to be fertile and to breed as non-yellow. Admittedly, the number of offspring was small and a more appropriate yellow stud could have been used. Thus, it appears most likely that the exceptional non-yellow females, which breed as homozygotes, receive a wild-type allele from a yellow heterozygous XXY sire; those which breed as heterozygotes are normal overlaps.

The hypothesis of Komai and Ishihara (1956) should be mentioned since it has been given more consideration than other explanations in recent years (Jude and Searle, 1957; Robinson, 1959; Gowen, 1961). Komai and Ishihara postulated that a male could possess two genes for the yellow locus if an X chromosome fragment bearing that locus had crossed over to the Y chromosome in his sire. If the son were heterozygous, he could show the tortoiseshell pattern. If proposed fertility genes were lost from the Y in the crossover process, the resulting son would be sterile regardless of color. If the fertility genes were retained in the Y, the son would be fertile even if he were tortoiseshell. Exceptional non-yellow daughters of yellow males would be produced when the deficient X combined with a wild-type allele from the dam.

The hypothesis of Komai and Ishihara is untenable for several reasons. (1) In appropriate matings, fertile tortoiseshell males should produce fertile tortoiseshell sons. No typical tortoiseshell sons were produced by the fertile tortoiseshell male reported by Bamber and Herdman (1932). The three adult tortoiseshell sons of the tortoiseshell male reported by Jude and Searle were apparently sterile. (2) An exceptional yellow female from a non-yellow sire, as well as a non-yellow female from a yellow sire, would be expected if a deficient X from those sires combined with X chromosomes bearing, respectively, a yellow or a wild-type allele from the dams. The writer has found no report of exceptional yellow females being sired by non-yellow males. (3) Nearly fatal to the hypothesis is the case of the yellow male siring 9 non-yellow in 17 daughters (Bonhote, 1915). According to the hypothesis, over 50 percent of the effective sperm would have had to contain X chromosomes from which a fragment had crossed over to the Y chromosome. The anomalous yellow male sired no tortoiseshell males; some would have been expected if the crossover percentage had been unusually high. The occurrence of more than one crossover type in a litter would be very unlikely, yet there are records of two litters, each of which contained two tortoiseshell males (Bonhote, 1915; Tjebbes and Wriedt, 1926). Bonhote obtained his data from a fancier, but Tjebbes and Wriedt's case was included in their own experiments.

Mutants in which the Tissue Environment Determines the Contrasting Colors Produced by the Melanocytes

Not all mutants which exhibit more than one color of pigment are mosaics in which groups of pigment cells differ in genotype. Thus, it is necessary to review the developmental characteristics which can be used to distinguish variegation spotting mutants from mutants in which the color produced by the melanocytes is dependent upon the tissue environment.

In the first major subheading, it was shown that piebald and variegation spotting are developmentally related; the shape and extent of the pigmented areas depend on the migration paths of the pigment cells. When a mutation for piebald spotting is combined with one for variegation spotting, the pigment cells of the irregular variegation spotting patterns become grouped into separate wild-type and mutant spots in the guinea pig (Wright, 1917), cat (Whiting, 1919) and mouse (Schaible, 1959). If a redistribution of the differently pigmented regions does not occur, then it can be concluded that the melanocytes are not acting autonomously to produce the pattern; that is, the tissue environment controls what type of pigment will be produced by the melanocytes. The reaction of a pattern type with piebald spotting can be used as a criterion for classification only if the assumption is made that the piebald spotting patterns are dependent on restricted migration of melanoblasts. Since the reaction was used in the proof of the assumption, it cannot be used as the sole criterion for classification of pattern types even though it may be valid. Fortunately, there are other criteria that can be used.

Any means of demonstrating that the melanocytes autonomously produce contrasting colors can be used as a criterion for classifying a pattern as a variegation spotting type. If gene action is autonomous in the melanocytes, the differently colored areas of a genetic mosaic will correspond exactly with the distribution of genetically different melanocytes. But if the gene produces its effect by action in the follicles, the differently colored areas will correspond with the distribution of follicles instead.

In the mouse, gene action has been shown to be locally determined for all color mutants that have been subjected to skin transplantation experiments; that is, neither the donor nor host skin affect the coat color of the other (Gluecksohn-Waelsch, 1951; Grüneberg, 1952). Autonomy of the melanocytes can be tested by transplanting them alone or by observing the effect of host pigment cells which migrate into a differently colored skin graft. Silvers (1961) found that the alleles of the non-agouti locus acted through the hair follicles rather than in the melanocytes in the determination of yellow versus black pigment. When pigment cells from a yellow ($\underline{A}^{y}$) host migrated into a graft from a genetically black ($\underline{a}$) donor, the host pigment cells produced black coloration in the hairs. Likewise, when the host was black and the donor was genetically yellow, the host melanocytes produced yellow coloration in the hairs of the graft. In all cases the donor graft carried another color marker so that the effects of the host pigment cells could be distinguished from those of the donor. The actions of the marker genes, albino ($\underline{c}$) and extreme dilution ($\underline{c}^{e}$), were shown to be autonomous in the

melanocytes. Brown (b) and pink-eyed dilution (p) have been shown to act autonomously in the melanocytes in similar experiments.

Lyon (1960) described a new mutant in the mouse which appeared to be a repeat mutation of sex-linked mottled (Mo). Patches of light colored and non-Mo fur graded into one another; individual hairs were variable in color. The vibrissae were curly, particularly when they were light colored. In addition to normal appearing melanocytes, many cells that were presumed to be colorless pigment cells were found in the follicles of the light colored regions of the coat. Lyon (1960, 1962) considered the effect to be very different from that of piebald spotting mutations. She indicated that a similar mutant, tortoiseshell (To) described by Dickie (1954), was probably allelic although no linkage tests had been made. Dickie observed no redistribution of the differently colored regions when To was combined with a piebald spotting mutation (s). It appears from the structural effects on the hairs and the lack of pattern changes in combinations with (s), that the mottled alleles act primarily through the follicles rather than autonomously in the melanocytes. Thus, the mottled mutants qualify as variegation but not as variegation spotting types. Dickie named her mutant "tortoiseshell" because it was sex-linked and exhibited a pattern somewhat like a tortoiseshell cat. However, tortoiseshell in the cat is a variegation spotting type while tortoiseshell in the mouse is not. Fortunately, mottled was described before tortoiseshell in the mouse; if tortoiseshell is proved to be allelic to mottled, it will assume the same basic symbol (Mo). If tortoiseshell proves to be qualitatively the same as Mo, even the name, which was an

unfortunate choice, can be dropped.

The bilateral regularity of the patterns in some mutants can be used to separate them from the variegation spotting types in which contrastingly colored patches are distributed nearly at random among the different pigment areas. The patterns governed by the non-agouti locus in mammals are good examples of the regular or "bicolor" types.

The non-agouti mouse (a) has black fur except for specific regions wherein the hairs are yellow (Hollander and Gowen, 1956). For some reason unknown to the writer, the yellow hairs surround regions in which there has been an invagination of the ectoderm, that is, around the mouth, ears, nipple areolae, and perineal region. Epidermal ridges are characteristic of the same locations except that hair follicles apparently take the place of the ridges in laboratory rodents (Montagna, 1956, p. 22). Rawles (1955b) discussed the possible inductive relationships between the mesoderm and ectoderm in the formation of feathers, hairs, and epidermal ridges. The yellow regions of the neck pattern of the pronghorn antelope, sketched on the cover of The Journal of Mammalogy, apparently correspond to the locations of embryonic gill slits and cervical fistulas; see sketches (Arey, 1954, p. 196). If so, the yellow throat markings also correspond to regions of ectoderm invagination. The transitory or permanent thinness of the ectoderm may be a more general condition of the yellow areas. In the wild-type pattern of cats, the skin in the yellow stripes was noted to be thinner than in the black stripes (Little, 1958).

In comparison to the non-agouti mutant, the yellow regions are

extended to include the entire ventral surface and feet in the black and tan ($\underline{a}^t$) mouse. An apparently homologous black and tan pattern, which is found in dogs, can be varied by selection from a solid black animal through the types described for the mouse to the point where only a black saddle is left on the back (Little, 1957).

Because the black and yellow regions are regularly located in $\underline{a}^t$ mutants, it could be predicted that the distribution of the yellow and black regions would not be altered in the least if a gene for piebald spotting were combined with $\underline{a}^t$. Pigmented spots appearing on the ventrum of an $\underline{a}^t$ mutant are yellow; spots on the dorsum are black. If a pigmented spot falls on the lateral junction between the black dorsum and yellow ventrum, the junction remains in the same place as in the $\underline{a}^t$ animal which is not spotted; see illustration of Beagle dogs (Little, 1957, p. 78).

Robinson's (1963) assumption that the yellow mutants of all mammals are homologous with the "$\underline{e}$" mutants was not well founded. Little (1958) had already shown that the cross of the sable ($\underline{a}^y$) Basenji X yellow Cocker Spaniel produced all black pups. The effects of the "$\underline{a}$" mutants are quite different in nature from the "$\underline{e}$" mutants. The effect of the $\underline{A}^y$ gene and its mutant alleles in the mouse is to widen or eliminate the subterminal yellow band of the agouti hair in specific regions of the body (Cleffmann,1963), the result is a bilaterally symmetrical, bicolor pattern of yellow and black regions. In contrast, the black and yellow patterns of the "$\underline{e}$" mutants are of the unsymmetrical variegation spotting type.

Methodology in Developmental Genetics

There is no technique by which melanoblasts can be identified unequivocally during the early phases of their migration out from the neural crest. According to one report, it appeared that melanoblasts of the chick embryo could be selectively stained with vital dyes even in the early stages; however, the identity of the cells was later contested (Romanoff, 1960, p. 1035). Transplantation of tissue from specific regions to the base of a wing bud of a differently colored host was used to determine the location of melanoblasts in the blastoderm (Rawles, 1940) and to determine the stage when melanoblasts arrived at specific sites in the embryo (Fox, 1949). Melanoblasts of the later stages have been identified in histological preparations by correlating the time of appearance of morphologically distinct cells with the time at which melanoblasts were included in a graft taken from the site. By that means, melanoblasts which appeared in the wing bud at 3-4 days and in the coelom at 6-7 days were identified respectively by Watterson (1942) and Reams (1956). By transplanting grafts to the coelom of the chick, Rawles (1947) was able to determine the stages when melanoblasts passed through various stages and arrived at the limb buds in the mouse. Although Rawles demonstrated that the melanoblasts were in the skin of the limb buds of the mouse embryo at 12 days gestation, Schumann (1960) could not identify melanoblasts in the skin of any region of the body prior to 13 days.

Methods from the field of developmental genetics can be used profitably to analyze the development of spotting patterns. The

principles involved are similar to those of experimental embryology, but the execution of the operations is dependent on mutant gene action. For a review of the many developmental problems that have been studied in mutants of the mouse, refer to Gluecksohn-Waelsch (1951, 1961).

A mutation can be used to consistently induce abnormal development at a specific stage and usually in a specific region of the body. Different individuals may then be sacrificed at successive stages to obtain a series of specimens in which the developmental progress of the resulting syndrome may be followed. In order to investigate an extensive physiological process, a number of different mutants may be needed to break the normal chain of reactions in many different places or to alter the same process in different parts of the body. Just as thorough a knowledge of the normal as the abnormal is obtained, since effects are determined by comparing the mutant with wild-type. For example, the absence of germ cells in a sterile mutant (W) of the mouse helped to determine the identity of migrating germ cells in normal embryos (Mintz and Russell, 1957). After genetic control has been demonstrated for a number of points in a developmental process, it becomes possible to predict with some accuracy where future mutations or accidents may interrupt normal development in other species as well as the one investigated.

Piebald spotting mutations, including several multiple allelic series, occur at more than 14 loci in the mouse. The restriction of pigment spread in the various mutants varies over a wide range. For example, only the toes and tail tips remain unpigmented in heterozygotes of W and Mi^{wh} but the coats of the homozygotes are completely white. Some mutants, such

as belt (bt) restrict pigment primarily in only one specific pigment area (Schaible, 1959). Since the mutants have such diverse effects, there is a wealth of material for comparative studies of gene effects and interactions in the development of complex patterns.

In addition to the consistent production of abnormal individuals, a genetic change may induce a developmental change in only a part of an organism. Comparison of the mutant with the normal cells in such a genetic mosaic reveals whether the cells behave according to their own genotype or are dependent on the tissue environment for expression of their characteristics. The value of genetic mosaics in the study of differentiation and other aspects of development has long been appreciated by geneticists working with Drosophila (Sturtevant, 1932; Patterson and Stone, 1938; Stern, 1955; Baker, 1963), but genetic mosaics have only rarely been considered in studies of development of mammals and birds.

Many principles of the methods used in experimental embryology are incorporated in the use of genetic mosaics (Stern, 1955). For example, a genetic change in a melanoblast during embryogenesis can be used to produce the same result as transplantation of melanoblast bearing tissue between embryos of different genotypes. Vital marking of cells with dyes or carbon particles is an experimental procedure used in the derivation of fate maps (Hamilton, 1952). Genetic marking of embryonic cells is comparable except that the cells cannot be traced until a phenotypic effect is produced by the genetic change. Nevertheless, utilization of mosaics has advantages over vital marking and transplantation in that genetic marking (1) is cell specific, (2) is not complicated by trauma

or the possibility of bacterial infection, and (3) requires no special technique or skill unless mosaicism is induced by treatment.

The determination of the lineage of cells contributing to the development of each part of the Drosophila eye was accomplished by the use of variegated mutants (Baker, 1963). The same method was used for determining the cell lineage in the pigment areas of the mouse coat in this study.

MATERIALS AND METHODS

Stocks

Necessity of special stocks

Silvers (1963) discussed the need for special stocks in studies of pigmentation patterns. The program which he described for the development of coisogenic color strains was similar to that used in this study.

Most stocks of piebald spotted animals are probably made up of a complex of major and minor genes similar to that of the s gene and "K" complex which Dunn and Charles (1937) analyzed in the mouse. They outcrossed their "all-white" line, which carried s and presumably most of the K genes in the homozygous state, and then backcrossed the wild-type allele of s into the all-white line. The s/+ segregates of some backcross generations were mated together. Although the ss segregates could be easily distinguished from the s/+ and ++ types in any one generation, all three phenotypes overlapped if all backcross generations were grouped together. The K complex resulted in a spotted phenotype when alone as well as in combination with s. Overlapping of phenotypes becomes particularly troublesome when factors of the K complex segregate in crosses subsequent to an outcross. Thus piebald spotting genes are usually considered to be poor genetic markers.

Because of the variation in piebald spotting patterns, mutations of the major gene are difficult to detect. Castle (1951) and Goodale (1937, 1942) originally attributed the results of their selection experiments on hooded rats and headdot mice respectively to mutation of

the major gene. In both cases it was later proved that the increased restriction of pigment areas was due to a combination of mutation and accumulation of gene complexes. When Russell and Russell (1959) used a piebald spotted stock in a specific locus test, they had to make extensive breeding tests in order to determine whether or not mutation had occurred at the s locus. Even then it was difficult to determine whether or not mutation to the same allele had occurred since expression of the marker varied so greatly.

The major piebald spotting genes should be much easier to work with if they were isolated from the minor genes of their respective stocks. Separation could be accomplished by repeated backcrosses of the major gene to a stock which was free of the minor genes. Since the desired stock could only be found in a classical geneticist's Utopia, the next best type of stock would be an inbred strain. If all of the experimental operations and breeding tests were carried out on an inbred strain, or possibly the final cross on an F_1 hybrid, the variability due to segregation of minor genes would at least be at a minimum. If the animals of the inbred strain had fully pigmented coats, as most wild-type animals do, then any piebald spotting pattern that was associated with the backcrossed mutant could be attributed mainly to the major gene. Certainly the differences among mutants could be attributed to single gene effects.

Two fully pigmented inbred strains which will produce vigorous F_1 offspring are needed. Inbreeding usually results in weaker animals than outbreeding or random breeding. Mutant animals are weaker than wild-type. Though the mutants are not easy to maintain in the coisogenic strains,

the homozygotes obtained by crossing mutants from the two strains are frequently superior to random bred homozygotes (Russell et al., 1962). If the coisogenic stock begins to wane, a cross to a hybrid female will give the stock a greater boost than a random outcross. If both inbred strains are fully pigmented, very few backcrosses to the original strain will be needed to stabilize the piebald spotting pattern again.

Formation of stocks

All stocks for this study were developed from multiple marker stocks and inbred strains maintained at the Genetics Laboratory of Iowa State University. Unfortunately, no fully pigmented inbred strains were available when this study was commenced in 1956. All strains carried the markers, albino (c) which eliminates all pigment or silver (si) which reduces the number of active melanocytes per follicle (Chase and Mann, 1960). In addition, many of the strains carried brown (b) which reduces the contrast between pigmented and unpigmented regions. One marker stock was found to be fully pigmented and highly inbred as a result of mating within a small closed population since 1950. From that stock the NAW strain was bred. Three additional fully pigmented strains (Z-c^+, MWA, and ENA) have been formed. The inbred and coisogenic strains have been named and described according to the standardized nomenclature for inbred strains of mice (Snell et al., 1960). All four strains have been selected for vigor and against pigment area restriction during inbreeding. Occasionally a few toes are white, usually on the rear feet if any.

Strain: NAW (non-agouti, waved)

Genetics: aa wa-2wa-2 The waved mutation, observed at the Rockefeller Institute of Medical Research by Dr. John W. Gowen (personal communication, 1962), appeared to be a repeat of wa-2. The allelism test was conducted by Dr. Clyde E. Keeler (unpublished).

Origin: marker stock (aa wa-2wa-2 vv male X aa wa-2wa-2 v/+ female) which had 59 percent of its background in common with the Z strain. Bxs matings since 1954 except for one pair which came from two litters produced by one male and two litter sisters in the 4th generation. After the 5th generation only non-waltzer (v^+) mice were mated.

Inbreeding: F26

Strain: Z-c^+

Genetics: Wild-type

Origin: The original W^a mutant male was sired by an X-rayed male from the Z strain (albino, c) and an a b p d se dam (Hollander, 1956). In order to maintain W^a on the genetic background in which it was induced, the original male was backcrossed to the Z strain. After 13 backcrosses of W^a c^+ to Z, an inbred strain was established by bxs matings of the wild-type segregates.

Inbreeding: N14 + F3

Strain: MWA - $+W^a$ (modifiers of W^a)

Genetics: $a^e a^e$ $W^a/+$

Origin: Crosses involving strains K, NAW, Z - +c $+W^a$ (N4) and yellow

male No. 5 in Fig. 15 of Hollander and Gowen (1956) yielded $\underline{a}^e\underline{a}^e$ $\underline{W}^a/+$ segregates which were selected for an even roan pattern and mated together for several generations. Then bxs matings of $\underline{W}^a/+$ X ++ were begun in which selection for even roaning was continued on the $\underline{W}^a/+$ parent, but the ++ parent was selected for no white. The 3rd generation of bxs matings involved a trio of littermates in the same cage; hence, the 4th generation pairs may not have been full sibs.

Inbreeding: F6

Strain: ENA - + $\underline{Mi}^{wh}$ (extreme non-agouti)

Genetics: $\underline{a}^e\underline{a}^e$ $\underline{Mi}^{wh}/+$

Origin: Cross of Goodale Large stock with $\underline{a}^e$ descendants of yellow male no. 5 in Fig. 15 of Hollander and Gowen (1956) yielded $\underline{a}^e$ F_2 offspring to which $\underline{Mi}^{wh}$ was backcrossed 3 times. Thereafter matings were bxs ($\underline{Mi}^{wh}/+$ X ++). The effects of many minor as well as major piebald spotting genes are revealed in combination with $\underline{Mi}^{wh}/+$. Inclusion of $\underline{Mi}^{wh}$ in the stock allowed for more effective selection for minimum restriction of pigment areas.

Inbreeding: F5

The piebald spotting mutants were backcrossed primarily to the NAW strain since it was well established and was not segregating albino. Usually the first few offspring of a backcross generation were used as parents for the next generation. When there was a choice, those showing minimum restriction of the pigment areas were selected as parents. $\underline{Mi}^{wh}$

was kept segregating in the matings to reveal the heterozygotes of the recessive piebald spotting mutants that were backcrossed. However, once the minor spotting genes and modifiers had been eliminated, the combination of $\underline{Mi}^{wh}$ with some of the recessive heterozygotes no longer resulted in restricted patterns. Thereafter F_2 generations were alternated with the backcross generations in order to obtain homozygous recessive mutants.

The NAW strain is low in vigor partially because it is homozygous for wa-2. Hence, only animals carrying the wild-type allele of wa-2 were used as backcross parents. Backcrossing progressed much faster, of course, for the dominant than for the recessive genes. After generation N10, the non-waved segregates which carried no piebald spotting gene were utilized as NAW parents for backcrosses that were more than 5 generations behind.

References for the origin and description of each mutant are listed annually in the Mouse News Letter. Since "headdot" (Keeler and Goodale, 1936) has not been given a symbol, it is not included in the lists. To the best of the writer's knowledge all mutants used in this study except bp, v, and wa-2 are the original alleles. From breeding tests and gross appearance the exceptions seem to be repeat mutations of the original alleles.

The following coisogenic strains were formed:

NWA - $+\underline{W}^{a}$ - $\underline{a}^{e}\underline{A}^{y}$ (N2)

NAW - +wa-2 +bt (N6)

NAW - +wa-2 +headdot (N4)

NAW - $+\underline{wa\text{-}2}$ $+\underline{mi}^{bw}$ (N6)

NAW - $+\underline{wa\text{-}2}$ $+\underline{Mi}^{wh}$ (N13)

NAW - +wa-2 +Q (N10)

NAW - +wa-2 +s (N7)

NAW - $+\underline{wa\text{-}2}$ $+S1^{gb}$ (N7)

NAW - +wa-2 +Sp (N11)

NAW - +wa-2 +Va (N9)

NAW - +wa-2 +W (N15)

NAW - +wa-2 +W^a (N14)

Z-c^+ - +W (N15)

Z-c^+ - +W^a (N17)

The restriction of pigment spread in each mouse of the inbred and coisogenic strains was recorded by scoring each pigment area as absent (-), restricted (r) or wild-type (+). The unpigmented fraction of the tail was estimated. In many types only a few toes remained unpigmented; a toe was recorded as white when there was no pigment distal to the proximal knuckles.

In the N11 to N14 generations of backcrossing W and W^a to both NAW and Z strains, heterozygotes within each coisogenic strain were mated together to determine whether or not increased pigment spread (presumably increased proliferation of melanoblasts) would result in greater viability of the homozygotes (presumably because of a correlated increase in the number of blood cells). The offspring of each mating were classified every 3 days. The litter was destroyed if there were no homozygotes. If there were homozygotes, the litter size was reduced to 3 normal mice in addition to the anemics; the entire litter was not destroyed until all of the homozygotes died. The matings were continued until at least 10 homozygotes had been classified in each coisogenic strain.

Prenatal mortality in matings of W and W^a heterozygotes within the NAW and Z coisogenic strains was investigated in generations N6 to N14. Matings were checked every 3 days; females which were obviously pregnant were palpated to determine the age of the embryos. The females were sacrificed if the embryos were 15 days or older. The fact that the toes

of the embryos are separate at 15 days but are nearly fused on the 14th and 16th days (Grüneberg, 1943) allowed for a more exacting check of age. The numbers of live, anemic, recently dead (eye spot apparent) and early dead (deciduomata) embryos were recorded.

In this study only the effect of each piebald spotting gene in its coisogenic strain will be described. In a later paper the effects of the combinations of spotting genes within the NAW strain will be presented in "interaction charts"; some of the required crosses have already been made. In this thesis those combinations involving $\underline{Mi}^{wh}$ will be discussed in relation to the effect on the frequency of mosaicism.

The markers of each linkage group were combined into separate stocks. All markers, regardless of dominance, were placed on one homologue and only the recessive markers on the other. The stocks are maintained by (1) crossing individuals having both chromosomes marked to an inbred strain, (2) obtaining F_2 offspring with both chromosomes marked, and (3) repeating the first two steps in backcrosses. Currently most of the linkage groups are being backcrossed to the NAW - $+\underline{wa\text{-}2}$ $+\underline{a}^{e}$ (N7) coisogenic strain. The groups which carry hair structure mutants are being backcrossed to ENA. As in the coisogenic spotting stocks, it would be ideal to have each marker group backcrossed to two inbred strains that combine well. The testcrosses could then be made up so that the offspring on their dams would be hybrids. Again, if a coisogenic marker strain began to die out, it could be established again by crossing to the hybrid and repeating the backcrosses to the inbred strain.

The assembling and backcrossing of the markers of each linkage group

had reached the following state by the end of the year 1962.

I NAW - $\underline{a}^{e}$ $\underline{wa\text{-}2}^{+}$ - $\underline{a}^{e}\underline{A}^{w}$ ($\underline{c}^{ch}$ $\underline{p}/\underline{c}^{ch}$ $\underline{p}$) (N2)

II NAW - $\underline{a}^{e}$ $\underline{wa\text{-}2}^{+}$ - $a^{e}a^{t}$ ($\underline{d}$ $\underline{se}/\underline{d}$ $\underline{se}$) (N1)

III NAW - +$\underline{wa\text{-}2}$ ($\underline{hr}$ $\underline{s}$ +/+ $\underline{s}$ $\underline{pn}$) (N1)

NAW - +$\underline{wa\text{-}2}$ +$\underline{W}^{a}$ (N14)

IV NAW - $\underline{a}^{e}$ $\underline{wa\text{-}2}^{+}$ - +$\underline{b}$ ($\underline{si}$ + $\underline{av}$/+ $\underline{Sl}^{gb}$ +) (N1)

V NAW - $\underline{a}^{e}$ $\underline{wa\text{-}2}^{+}$ - ($\underline{bp}$ $\underline{A}^{y}$ $\underline{pa}$ $\underline{Sd}/\underline{bp}$ + $\underline{pa}$ +) (N1)

VI ENA - ($\underline{N}$ $\underline{Ca}$ $\underline{hl}$ $\underline{bt}$/+ + $\underline{hl}$ $\underline{bt}$) (N1)

VII ENA - ($\underline{Re}$ $\underline{df}$/+ +) (N2)

NAW - ($\underline{df}$ +/+ $\underline{wa\text{-}2}$) (N6)

VIII NAW - $\underline{a}^{e}$ $\underline{wa\text{-}2}^{+}$ - ($\underline{m}$ $\underline{Pt}$ $\underline{b}/\underline{m}$ $\underline{Pt}$ $\underline{b}$) (N2)

IX NAW - +$\underline{wa\text{-}2}$ +$\underline{T}$ (N3)

NAW - +$\underline{wa\text{-}2}$ +$\underline{Fu}$ (N3)

X NAW - +$\underline{wa\text{-}2}$ $\underline{aA}^{y}$ +$\underline{v}$ (N2)

XI ENA - ($\underline{Mi}^{wh}$ $\underline{wa\text{-}1}$/+ $\underline{wa\text{-}1}$) (N2)

XII NAW - $\underline{a}^{e}$ $\underline{wa\text{-}2}^{+}$ - ($\underline{ru}$ $\underline{je}/\underline{ru}$ $\underline{je}$) (N1)

XIII ENA - ($\underline{ln}$ $\underline{Sp}$ $\underline{fz}/\underline{ln}$ + $\underline{fz}$) (N1)

XIV NAW - +$\underline{wa\text{-}2}$ +$\underline{f}$ (N3)

XVI NAW - $\underline{a}^{e}$ $\underline{wa\text{-}2}^{+}$ - +$\underline{Va}$ (N7)

XX NAW - +$\underline{wa\text{-}2}$ +$\underline{To}$ (N6)

This method of maintaining linkage stocks is particularly advantageous for linkage studies of piebald spotting mutants. All markers are on the same genetic background and easier to classify than in heterogeneous stocks. A new mutant is immediately crossed and backcrossed to the two inbred strains or coisogenic marker strains. The linkage groups that are

easiest to classify can be worked on first; those groups in which overlap of phenotypes is more likely can be attempted after the new mutant has been backcrossed several times. The first cross with any group immediately gives repulsion heterozygotes for multiple-point testcrosses; that is, the mutant is on the homologue opposite the markers. The multiple recessive testcross parent is derived from the first cross offspring which carry the recessive markers of the group on one homologue and the new mutant on the other, providing the new mutant is recessive. If the new mutant is dominant, the multiple-recessive testcross parent, of course, is derived solely from the recessive markers.

From the standpoint of detecting new mutations, it is advisable to have the piebald spotting loci flanked on both sides by other markers as in the case of hr s pn in group III, si Sl^{gb} av in group IV, and ln Sp fz in group XIII. In specific locus tests this arrangement should facilitate the detection of chromosomal aberrations and the separation of induced mutants from known markers in breeding tests. In cases where gene conversion is suspected, both sides of the locus must be marked. When a locus that is marked on both sides mutates, contamination can be ruled out. Contamination becomes a real problem when several similar alleles are maintained in one laboratory (Ballantyne et al., 1961). Three of the groups carry piebald spotting mutants that are marked on one side; hl bt in group VI, m Pt in group VIII, and Mi^{wh} wa-1 in group XI.

One bt stock and two s stocks, all three of which contained complexes of minor piebald spotting genes, were selected for particular patterns.

The selection experiments with s resulted in "white-face" and "white-body" stocks which are similar to those described by Dunn and Charles (1937). In the present experiment one parent of each mating was always maintained heterozygous for Mi^{wh} in order to detect the presence of subthreshold piebald spotting genes. Matings were generally made within families showing the most desired type rather than by selecting exceptional individuals from separate families. The selection criteria in the order of emphasis and references to photographs of typical selected breeding animals are given in the following accounts of each stock.

MBT (modifiers of bt), Figs. 12 and 13

1. Complete pigmentation in all regions of the skin except the lumbar and adjacent costal and sacral areas.
2. Absence of pigment centers in the lumbar areas.
3. Maximum restriction of the costal and sacral pigment areas.

MSC (coronal modifiers of s), Figs. 14 and 15

1. Absence of the coronal pigment center and others more anterior.
2. Minimum restriction of the lumbar pigment areas and others more posterior.

MSL (lumbar modifiers of s), Figs. 19 and 20

1. Absence of the lumbar pigment centers and others more posterior.
2. Minimum restriction of the pigment areas of the face.

Plate 2. Selected piebald spotting stocks

MBT stock (*a*^{e} *bt*). Complete restriction of lumbar pigment areas

Fig. 11. Usual width of belt

Fig. 12. Restriction of adjacent costal pigment areas as a result of selection for increased width of belt

Fig. 13. Costal areas reduced to centers by adding *Mi*^{wh}/+ to a genotype like that of the mouse in Fig. 12

MSC stock (*a s*). Complete restriction of the facial pigment areas

Fig. 14. *Mi*^{wh}/+ white-face type; all pigment centers present and restricted on body; facial and aural pigment areas absent

Fig. 15. "Ideal" white-face type on left. Extreme restriction in *Mi*^{wh}/+ white-face type on right; incomplete primary breaks between sacral and caudal areas

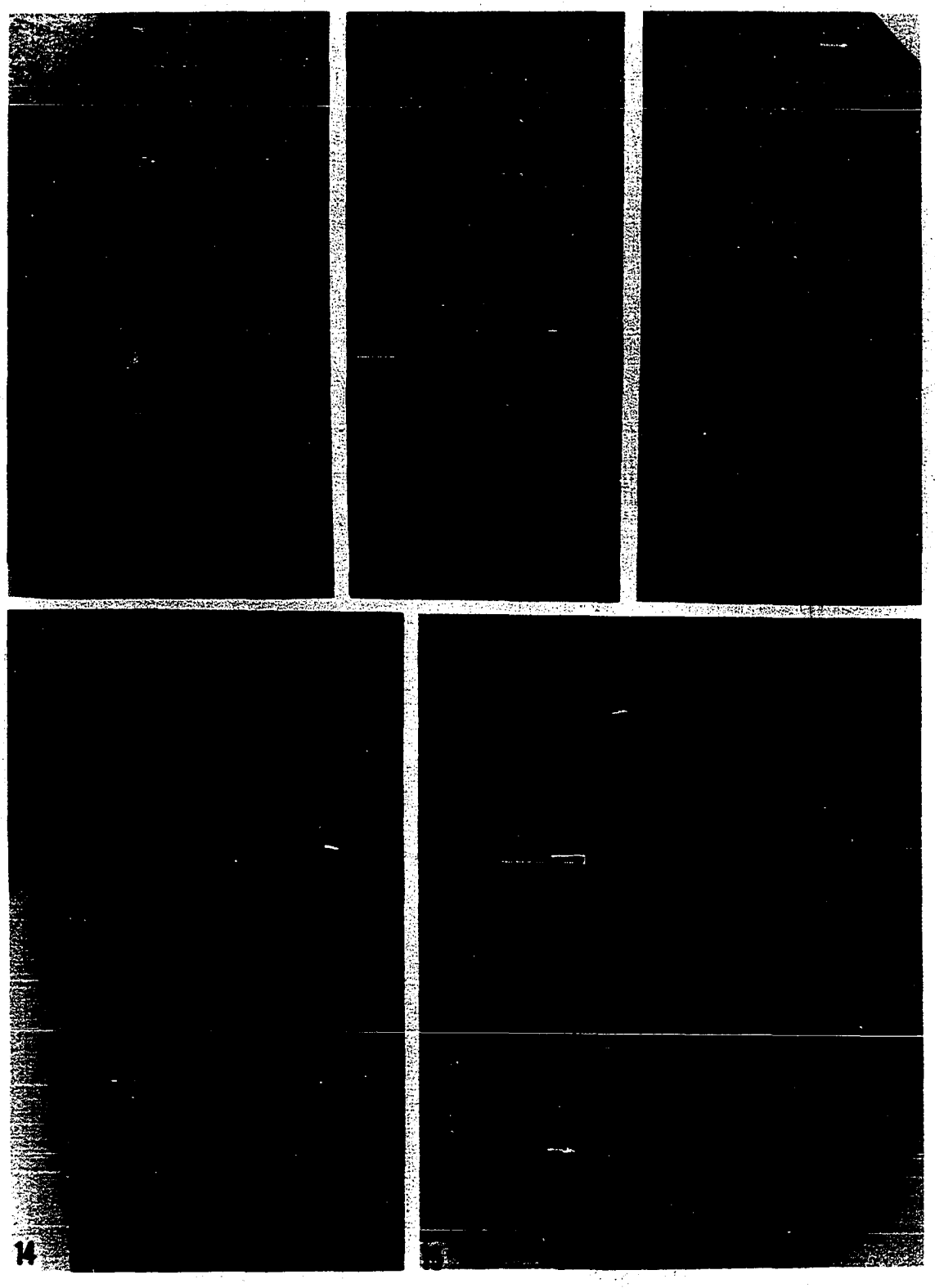

14

Plate 2. (continued)

MSL stock (a s). Complete restriction of the lumbar pigment areas. All illustrated mice except for the one on the right in Fig. 20 are $\underline{Mi}^{wh}/+$

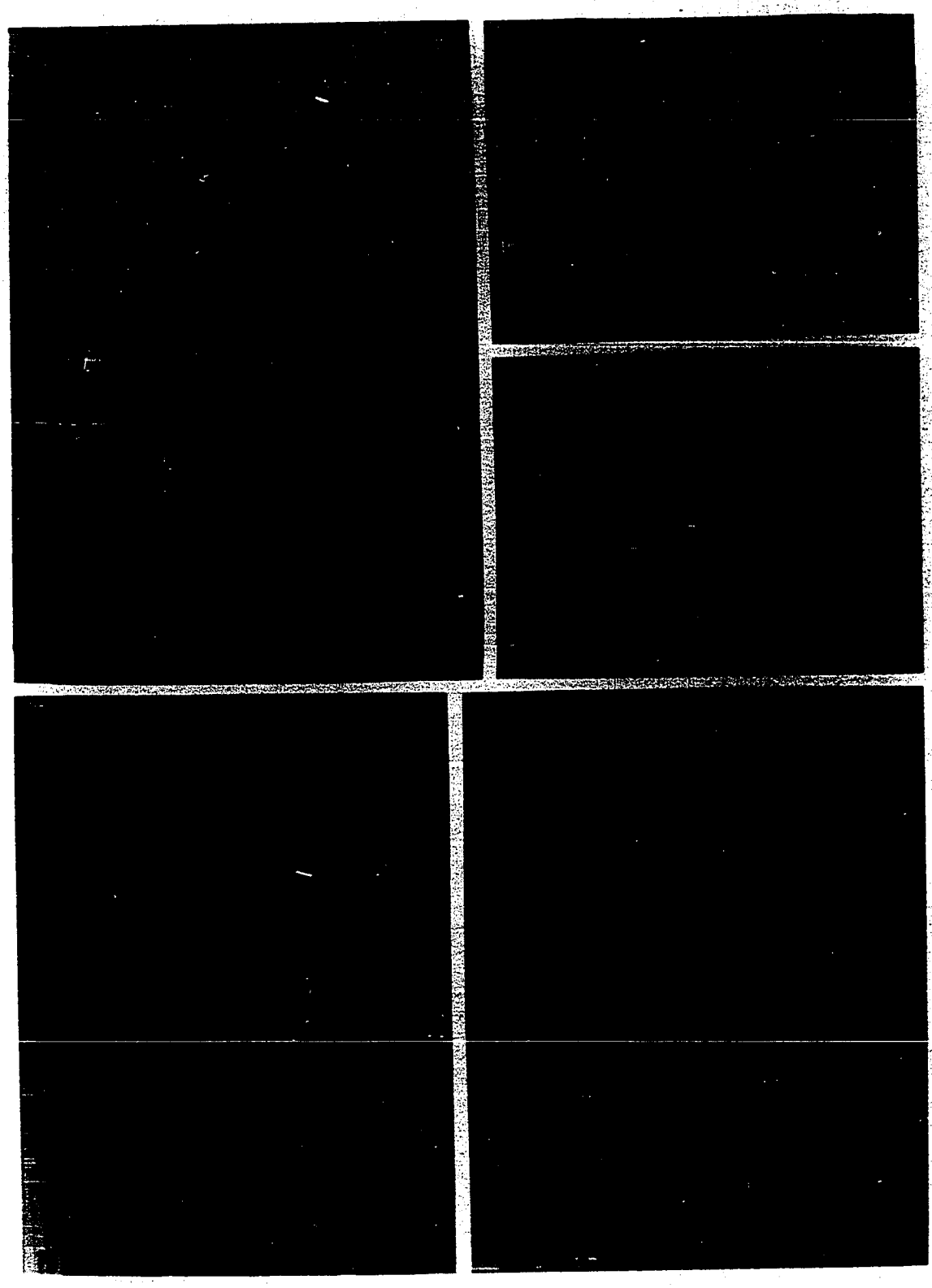
Fig. 16. Restricted coronal area shown to be separate from aural and temporal centers

Fig. 17. Restricted sacral areas

Fig. 18. Development of only the coronal area

Fig. 19. Type selected as $\underline{Mi}^{wh}/+$ parent in stock; all pigment areas present on fact but none on ears or body

Fig. 20. Non-$\underline{Mi}^{wh}/+$ type on right used as parent in selected stock. Coronal area only in $\underline{Mi}^{wh}/+$ type on left



Experiments on Frequencies of Mosaicism

Various crosses were set up to test different agents which might affect the frequencies of mosaicism. Segregation ratios of alleles were used to test for germinal mosaicism. The methods given in chapter nine of Snedecor's (1956) text were used for all statistical analyses.

The mosaics of all the $\underline{Mi}^{wh}$ and $\underline{Va}$ crosses were classified between 9 and 30 days of age. The juvenile coat is growing on all parts of the body at 9 days; the second coat begins growing at about 30 days (Borum, 1954). Mosaics of $\underline{Mi}^{wh}$ heterozygotes are easier to classify in juvenile than in adult coats. The coat darkens with age; the contrast between $\underline{Mi}^{wh}/+$ and non-$\underline{Mi}^{wh}$ areas is reduced. The variegation patterns of $\underline{Va}$ mutants appear to be different in the juvenile and adult coats. The dorsal and ventral color patterns of each mosaic were sketched on an outline similar to that of Fig. 7.

Influence of piebald spotting mutants

In the early stages of establishing the inbred and selected stocks, the following crosses and their reciprocals with regard to sex were made to determine the influence of $\underline{s}$ and $\underline{bt}$ on the frequency of genetic change in $\underline{Mi}^{wh}$ heterozygotes. Because $\underline{Mi}^{wh}$ primarily dilutes black rather than yellow pigment, all mice involved in the crosses were either non-agouti ($\underline{a}$) or extreme non-agouti ($\underline{a}^{e}$).

$\underline{Mi}^{wh}\underline{Mi}^{wh}$ X ++

$\underline{Mi}^{wh}\underline{Mi}^{wh}$ X $\underline{ss}$

$\underline{Mi}^{wh}\underline{Mi}^{wh}$ X $\underline{btbt}$

$\underline{Mi}^{wh}\underline{Mi}^{wh}$ $\underline{ss}$ X ++

$\underline{Mi}^{wh}\underline{Mi}^{wh}$ $\underline{btbt}$ X ++

$\underline{Mi}^{wh}/+$ X $\underline{Mi}^{wh}/+$

$\underline{Mi}^{wh}/+$ $\underline{ss}$ X $\underline{Mi}^{wh}/+$ $\underline{ss}$

$\underline{Mi}^{wh}/+$ $\underline{btbt}$ X $\underline{Mi}^{wh}/+$ $\underline{btbt}$

Data for the following crosses and their reciprocals with regard to sex were obtained from the backcross and selection stocks.

$\underline{Mi}^{wh}/+$ X ++ $\underline{Mi}^{wh}/+$ btbt X btbt

$\underline{Mi}^{wh}/+$ ss X ss

Influence of irradiation during pregnancy

Pregnant females of the cross $\underline{Mi}^{wh}\underline{Mi}^{wh}$ male X btbt female were X-rayed at different stages during the first 11.5 days of pregnancy to determine the effect of radiation on the frequency of mosaicism. Pregnancies were timed by checking the females each morning for vaginal plugs. Eleven days after the first plug was found, all the females in which plugs had been observed were given 150r to the region of the uterus at a time late in the morning. Because mice usually breed between 10 P.M. and 1 A.M. (Snell, 1941), it was assumed that the X-ray treatment was applied at approximately 12 hours plus whole day intervals following copulation.

The mice were X-rayed on a wooden platform and restrained under arches of celluloid sheets which fitted into grooves on the platform. The head, thorax, and tail were shielded with lead plates. The radiation specifications were made to approximate those of Russell and Major (1957) as closely as possible, so that the results could be compared. The dose was administered with a General Electric Maxitron (250 p.kv., 30 ma., 2mm. Al filters) 84 cm. from the subjects at a rate of 85-89r/min. The animals were treated in 5 different batches during the period, Jan. 9 - Aug. 17, 1958. The last batch accounted for approximately half of the total. Litters from females which bred but showed no plugs were included

among the controls.

All litters from X-rayed dams were classified by 14 days of age, since there were induced abnormalities which increased mortality rates. Presence or absence of dorsal and ventral restriction of the lumbar pigment areas was recorded.

Tests for twin spots

Double heterozygotes of *Mi*^{wh} and *wa-1* were examined for waved hair mosaics as well as color mosaics. Four percent recombination was listed by Green (1963) for the two loci. Some of the *ss* parents in the *Mi*^{wh}*Mi*^{wh} X *ss* cross were homozygous for *wa-1*; those matings produced offspring in which the mutant genes were in repulsion (*Mi*^{wh} +/+ *wa-1*). Heterozygotes having mutant genes in coupling (*Mi*^{wh} *wa-1*/+ +) came from the cross *Mi*^{wh} *wa-1*/+ *wa-1* X ENA in the group XI linkage stock. Both hair structure and hair color mosaics including twin spots could be expected if mitotic crossing-over, mitotic nondisjunction, mitotic disjunction or mitotic reduction were involved. The mice were held between the eye and a light source and rotated in order to view the coat over most of the body. The straight hairs of normal 9-21-day-old mice show an even halo effect when viewed in such a manner, but waved hairs do not. Small mosaic regions consisting of a few hairs could be missed but large regions would be detected.

Tests for germinal mosaicism

Initially each *Mi*^{wh}/+ mosaic, whether *s*/+, *bt*/+ or free of *s* and *bt*, was mated to the ++ segregates of the *Mi*^{wh}/+ X *Mi*^{wh}/+ cross to test the

segregation of $\underline{Mi}^{wh}$ and +. Offspring of those mosaics which carried neither bt nor s were used to test subsequent $\underline{Mi}^{wh}$/+, non-bt, non-s mosaics. After several generations each animal of the tester stock (Mos) had a large number of mosaics in its pedigree. Mosaic offspring of the test matings were also tested. Initially all mosaics were tested, but as the numbers of them increased, testing was limited to those exhibiting the largest altered areas. The test matings in the Mos stock should have shown a high frequency of mosaicism if selection had any effect. Mosaic offspring of $\underline{Mi}^{wh}$/+ bt/+ or $\underline{Mi}^{wh}$/+ s/+ mosaics were assumed to be free of bt or s if their own $\underline{Mi}^{wh}$/+ offspring showed no dorsal restriction of the pigment areas on the body. Nevertheless, the supposedly bt and s free matings were recorded separately as bt^{+} Mos and s^{+} Mos stocks.

By the same methods as given above for $\underline{Mi}^{wh}$, a tester stock ($\underline{W}^{a}$ Mos) was developed for the $\underline{W}^{a}$/+ mosaics.

No mosaics were used as parents in any crosses or stocks other than tester stocks except when Va was involved. Since nearly every Va mutant is a mosaic, no effort was made to select for or against mosaicism.

An attempt was made to obtain 50 offspring from each mosaic. Females were tested in single pair matings and males in trios. If a mosaic produced a ratio which deviated significantly from 1:1 at the .05 probability level, 50 more offspring were obtained when possible.

Progeny testing for recessive genes

Occasionally the $\underline{Mi}^{wh}$/+ mosaics exhibited areas in the coat which were not typical of the effect of the wild-type allele. Some off-color

areas were a darker and some a lighter shade than the heterozygote (Figs. 38 and 39). The effect could have been due to a mutation at another locus, particularly if a recessive color marker were segregating in the cross. Most of the stocks had other color markers in their ancestry. The anomalous mosaics were tested for any recessive they might carry by breeding them to an appropriate number of full sibs or offspring (Kidwell and Ternan, 1957). When the mosaic died before breeding tests on it could be completed, testing, in some cases, was continued by mating trios of its offspring together. When possible, testing was carried to the point that 99 percent of the recessive genes carried by the mosaic should have been made homozygous in at least one progeny for each gene. The basis for determining the required number of mates and offspring per mate was presented by Falconer (1949).

$$p = [(1-h) + h(3/4)^s]^n$$

where p = probability of failure to detect that the individual is heterozygous.

(1-h) = probability that the mate (sib or progeny) is not heterozygous.

$h(3/4)^s$ = probability that the mate is heterozygous but fails to produce a recessive type progeny. s = litter size.

n = number of mates.

Breeding to offspring instead of sibs has the advantage of detecting new mutations carried by the individual as well as those carried by its parents. If the parents of the individual were treated with a mutagen, it is imperative that the individual be tested with its offspring. In

either case h = 1/2.

The same formula can be used to determine the probability that a recessive gene from a heterozygous individual will not be revealed in full sib matings of its offspring. h becomes the probability that both mates are heterozygous. When a mosaic of this experiment was tested by mating trios of its progeny, h became the probability that the male and at least one female mate were heterozygous (h = 3/8). A negative result of the test indicated that the mosaic parent of the mates did not carry a recessive mutant gene. Production of a recessive mutant did not prove that the mosaic carried the mutation since the mates could have received it from their other parent.

Since litter size usually varies among the test matings, the value within the brackets is usually calculated for each mating; then p equals the product of the values for each of the matings. Prior to testing, the required number of mates can be determined by solving for n if a conservative estimate of litter size (s) is made and a probability value (p) is set. The estimate of litter size must be conservative since a large litter from one mating cannot fully compensate for a small litter from another. The other alternative is to repeat each mating until the required number of offspring is produced.

In direct tests of mosaic individuals in this study, all estimates of p are high. The gene frequencies of the recessive markers were not taken into account in determining the probability that tester mates were heterozygous. The estimate of p is conservative in the sense that the tested mosaic is more likely to be free of recessive genes than the

confidence level indicates.

Production of homozygotes and compounds of Mi^{wh}, mi^{bw}, W^a, and Va

An attempt was made to obtain homozygotes of Mi^{wh}, W^a and Va with pigment in their coats; all three types are usually black-eyed whites. Homozygotes of W^a usually die within five days following birth. Since the viable homozygotes of some other W alleles are black-eyed whites, it is assumed that the homozygotes of W^a would be the same. Homozygotes of Va were reported to have some pigment near the ears and base of the tail (Cloudman and Bunker, 1945). If pigmented spots could be obtained in the coats of homozygotes, it should be possible to detect mosaicism and thereby determine whether or not genetic change was taking place in homozygotes as well as heterozygotes. Mutants that had been backcrossed to the inbred strains at least four times were used to obtain homozygotes. It was anticipated that the genetic background of the fully pigmented strains would allow for greater spreading of pigment in the homozygotes.

In testing for allelism of Mi^{wh} and mi^{bw}, Dr. Willard F. Hollander (unpublished) observed that the compounds (Mi^{wh}/mi^{bw}) exhibited light yellow spots even though homozygotes of Mi^{wh} never showed any pigment in their coats and mi^{bw} homozygotes very infrequently showed spots of normally colored fur. In this study Mi^{wh}/mi^{bw} compounds were obtained first in combination with non-agouti and then with extreme non-agouti (a^e) and examined for mosaicism. The spots were yellow even on a^e mice which ordinarily show no yellow pigment at all. Therefore, the compounds were also obtained in combination with yellow (A^y) to see if more pigment would be produced. The wild-type allele of A^y was kept in association

with bp, a closely linked marker, so that the yellow mutants (+A^y/bp +) could be distinguished from non-yellow (bp +/bp +) even in black-eyed white animals.

Experiment on Pigment Spread during Development of the Chicken

Spreading of pigment was compared among the Ancona, Black Polish Crested and Black Leghorn breeds. All eggs and chicks were purchased from MacMurray Hatchery, Webster City, Iowa.

Ancona chickens carry two mutations, extension of black pigment (E) and "mottled" (mo) (Hutt, 1949), which result in temporary restriction of the pigment areas in the down and juvenile plumages. Incubated eggs were X-rayed in attempt to obtain chicks which showed even greater restriction of the pigment areas than is characteristic of the breed. Russell and Major (1957) reported that pigment spread into the midventrum of C57BL mice was decreased by in utero X-raying at 10 1/4 days gestation. Retarded spreading of pigment in Black Minorca chicks was observed by Landauer (1954) as an effect of treating eggs at 96 hours incubation with various teratogenic agents.

Details of the experiment will be omitted for two reasons. (1) Faulty operation of the incubator allowed the eggs to cool during the 3rd and 4th days of incubation. As a result many embryos died; hatching was two days late for those that did survive. Because of retardation, the stages of development at the time of X-raying could not be accurately determined. Chilling and the X-ray treatment were confounded. (2) Variation in the extent of pigment spread proved to be so great in Ancona

chicks that very large numbers of them would have had to be examined to obtain a definite effect of treatment.

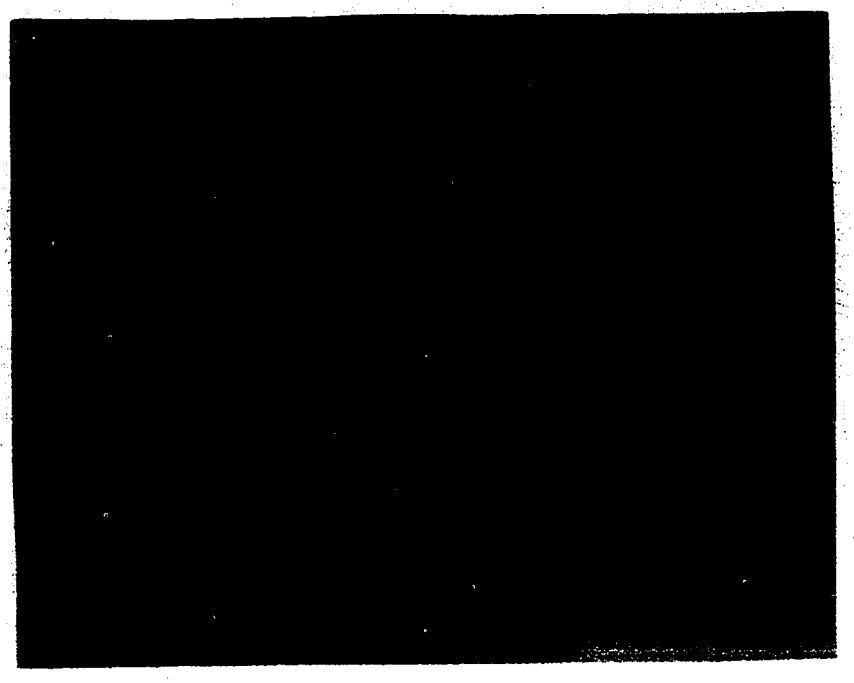
Nevertheless observations were made on 34 Ancona chicks that developed to the time of hatching; 26 were from the experiment in 1960 and 8 from other hatches. The spotting patterns were sketched on outlines similar to that of Fig. 43.

Ten chicks from the experiment were observed until all of the adult remiges had completed growth. Remiges of the juvenile and first adult plumage were collected from the three Anconas which showed the greatest restriction of pigment spread in the down pattern; two of them are illustrated in Figs. 21 and 42. Juvenile primary feathers were also collected from Black Polish Crested and Black Leghorn breeds (Figs. 22 and 41); both breeds are EE in genotype. When the base of a feather became translucent, it was cut off through the cornified region, rather than plucked, so there would be no damage to the next feather generation.

The growth and pigmentation of each juvenile and first adult remex of the 10 surviving chicks were recorded on forms as shown in Table 13. The first appearance of pigment in each follicle was described by recording (1) the date of observation, (2) the stage: down, juvenile or adult, (3) the length of the feather, and (4) the length of the pigmented portion of the feather. In the remaining columns the growth period of each juvenile and adult feather was noted by (1) the date that feather growth was first observed, (2) the length of the feather at the first observation, and (3) the date when the base of the feather became translucent. The lengths recorded were estimates in inches of the

Fig. 21. Extreme restriction of the pigment areas in the juvenile plumage of the Ancona chicken

Fig. 22. Juvenile plumage of the Black Polish Crested chicken



unsheathed portion of the vane. When only a pinfeather was present, it was so noted (p).

The growth data were utilized in making up charts on which the feathers were mounted. For example, the data of Table 13 were used in making the chart of Fig. 42. The dates of the observations are recorded on the horizontal lines of the chart. Each column represents the follicle of one remex. The juvenile and first adult feathers are, of course, in the upper and lower positions respectively. The length of a feather at the time it was first observed determined how far above the line the tip of the feather should be placed. The distance between observation lines was determined by the average growth of all the feathers between observations. Since the feathers grew at different rates, the time at which a feather stopped growing cannot be accurately determined from the chart. Still the relative positions of the feathers on the chart aid in tracing pigment spread with respect to both time and follicle location.

RESULTS

Pigment Spread in Special Stocks of Mice

Each piebald spotting mutant is described as it appeared in each specific strain and in combination with $\underline{Mi}^{wh}/+$ in some of the strains. Pigmentation of the feet and tail is so light in $\underline{Mi}^{wh}/+$ animals that a pattern could not always be detected and is not always reported. All of the coat can be assumed to be pigmented unless noted otherwise.

NAW-+wa-2+bt (N6)

In combination with $\underline{Mi}^{wh}/+$, homozygous bt resulted in a narrow, often incomplete belt. In non-$\underline{Mi}^{wh}/+$ segregates, there usually was no phenotypic effect of homozygous bt. The following ratio was produced from matings ($\underline{Mi}^{wh}/+$ bt/+ X bt/+) in the N2-N5 generations: 127 $\underline{Mi}^{wh}/+$: 31 $\underline{Mi}^{wh}/+$ belt:7 belt:140 showing neither mutant effect. Within the $\underline{Mi}^{wh}/+$ classes there was a reasonably good fit for the segregation of bt in a 3:1 ratio, but in the non-$\underline{Mi}^{wh}/+$ classes there was a definite shortage of belt phenotypes. The phenotypes of the 7 belt segregates clearly indicated that the phenotype of the non-btbt classes overlapped the homozygous bt class; the belt in all 7 segregates consisted of only a few white hairs located in the dorsal lumbar region.

NAW-+wa-2+f (N3)

Although "flexed" homozygotes tended to have more white toes than the NAW strain, positive identification was dependent upon anemia during the first three days following birth. White ventral regions and kinky

tails were characteristics of the original stock, but those traits were lost in the first backcross to the NAW strain. Combining $\underline{Mi}^{wh}$/+ with ff did not aid in identification since the feet of $\underline{Mi}^{wh}$/+ mice are all lightly pigmented.

NAW-+wa-2+headdot (N4)

In the N3 and N4 generations the penetrance of headdot was very similar to that of belt in that the homozygous headdot class was overlapped by the non-headdot class except in $\underline{Mi}^{wh}$/+ segregates. Double heterozygotes of headdot and $\underline{Mi}^{wh}$ often showed incompletely pigmented coronal areas; this phenotype overlapped the $\underline{Mi}^{wh}$/+ headdot homozygotes which had white coronal areas and usually restricted nasal and temporal areas. Both the headdot homozygotes and heterozygotes that were $\underline{Mi}^{wh}$/+ frequently showed primary breaks between the pigment areas on the belly. There was some selection for maximum restriction of pigment areas; the animals with the most white could be relied on to be headdot homozygotes and were used as backcross parents.

NAW-a^{e}-+wa-2-(m Pt b/m Pt b) (N2)

Four misty (m) segregates in the F_2 of the N1 generation showed white only on the toes primarily of the rear feet. The pigmentation of the brown misty segregates was too light in shade to allow for a distinct piebald spotting pattern on the feet.

NAW-+wa-2+Mi^{wh} (N13), NAW-+wa-2+mi^{bw}(N6) and $Mi^{wh}mi^{bw}$ in crosses

Not one of the 68 $\underline{Mi}^{wh}$ heterozygotes classified in the N12 and N13 generations showed any white in the coronal or belly regions even though

heterozygotes often did in outcrossed stocks. Their eyes had red pupils. As much as one-sixth of the distal part of the tail remained unpigmented in some animals but varied to complete pigmentation in others. Homozygotes were pure white.

The $\underline{mi}^{bw}$ allele was introduced into the NAW strain by repeated backcrosses of $\underline{mi}^{bw}/\underline{Mi}^{wh}$ to $\underline{Mi}^{wh}/+$ segregates of the NAW-$\underline{Mi}^{wh}$ coisogenic strain. The eyes of the compound type ($\underline{Mi}^{wh}/\underline{mi}^{bw}$) had red pupils; the juvenile coat was white with faint yellow spots. The yellow spots were replaced with white hair in the adult coat. A total of 177 compounds was observed; some including 12 $\underline{A}^{y}/+$ mice, were from crosses. The light yellow spots on the $\underline{A}^{y}/+$ mice did not appear to be darker or more extensive in size than the spots on the $\underline{a}$ or $\underline{a}^{e}$ mice. One compound showed microphthalmia; otherwise the eyes were normal except for a red pupil which is a characteristic of $\underline{Mi}^{wh}$ heterozygotes. The yellow spots often showed a slight sooty tinge, but the only possible indication of mosaicism in the compounds was the presence of a small spot of black hairs at the base of the left ear of one male.

No $\underline{mi}^{bw}$ homozygotes were produced in the NAW coisogenic strain, but in the C3HB/St strain and outcross stocks they were black-eyed whites which infrequently exhibited dorsal spots of apparently normal pigment intensity. The spots persisted through several molts.

NAW-+wa-2+Q (N10)

"Quinky" was characterized by the pleiotropic effects of kinky tail and choreic behavior in the Q strain in which it arose. The choreic behavior was not expressed by the mutant in the NAW coisogenic strain.

Only 12 out of 28 mutants had a kinky tail in the N8 - N10 generations. Quinky mutants could usually be identified by the criterion that most of their rear toes remained unpigmented. Quinky was no easier to identify in combination with $\underline{Mi}^{wh}/+$, since the latter gene caused the feet to be lightly pigmented. Of course the white toes could not be detected in the albino Q strain, but two backcrosses of $\underline{c}^{+}$ into the Q strain revealed the white rear toes on the Quinky segregates. Quinky homozygotes apparently died between implantation and 13 days gestation in heterogeneous stocks. Heterozygous males crossed to heterozygous females and their non-Quinky sisters produced 32.1 percent (54/168) and 6.2 percent (10/162) dead embryos respectively. If 6.2 percent of the normal embryos and 32.1 percent of all the embryos died when both parents were heterozygous, then the percentage of lethal homozygotes (X) can be obtained from the following equation:

$$6.2(100 - X) + X = 32.1$$

$$X = 27.6 \text{ percent}$$

NAW-+wa-2+s (N7)

The homozygous piebald segregates of the N4 to N7 generations were relatively uniform in pattern. The specific regions of pigment restriction are given in Table 1. In addition the feet were white in all homozygotes. In the $\underline{Mi}^{wh}/+$ $\underline{ss}$ segregates there were primary pigment breaks between most of the pigment centers. The breaks were the widest ventrally but also appeared consistently in the dorsal costal-lumbar region. In addition to the coronal area, one or both nasal areas were

Table 1. Number and percentage of animals of each sex which exhibited restriction of pigment in specific regions of the coat

Strain	Non-piebald spotted M	Non-piebald spotted F	Piebald spotted M	Piebald spotted F	Coronal restriction M	Coronal restriction F	Ventral restriction M	Ventral restriction F	Caudal restriction M	Caudal restriction F
NAW-s (N4-N7)			15	17	15	14	5	5	13	6
					100%	82%	33%	29%	87%	43%
NAW-Va (N4-N10)	63	81	62	61	62	61	62	61	26	22
					100%	100%	100%	100%	42%	36%
NAW-W (N1-N8)	161	134	146	122	16	8	17	8	146	122
					11%	7%	12%	7%	100%	100%
(N9-N15)	159	171	142	157	0	0	0	0	142	157
									100%	100%
Z-W (N1-N8)	22	25	14	30	0	0	1	4	14	27
							7%	13%	100%	90%
(N9-N15)	34	39	40	47	0	0	0	0	36	33
									90%	70%
NAW-W^a (N1-N8)	69	60	66	64	66	64	16	5	66	64
					100%	100%	24%	9%	100%	100%
(N9-N14)	61	77	92	72	92	72	7	0	92	72
					100%	100%	8%		100%	100%
Z-W^a (N2-N8)	36	71	68	66	62	56	28	21	68	66
					91%	85%	41%	38%	100%	100%
(N9-N17)	97	176	125	164	119	145	11	8	125	163
					95%	88%	9%	5%	100%	99%

frequently unpigmented in the $\underline{Mi^{wh}}$/+ $\underline{ss}$ animals.

NAW-~~twa~~-2+$S1^{gb}$ (N7)

"Grizzle-belly" arose in an F_2 female of the N1 cross of the $\underline{bt}$ mutant to the NAW strain. The F_2 female must have been a germinal mosaic; she did not show the characteristic herself and produced only 11 of the

grizzle-belly type in 37 offspring. Homozygotes of $\underline{Sl}^{gb}$ are anemic at birth and die within a week. The belly is grizzled and most of the toes are white in heterozygotes. The coronal area remains unpigmented in the double heterozygote of $\underline{Sl}^{gb}$ and $\underline{Mi}^{wh}$; the tail and belly are so lightly pigmented that the piebald spotting pattern cannot be observed in those regions.

NAW-+wa-2+Sp (N11)

Most of the toes were white in the 27 presumed "Splotch" heterozygotes of the N9-N11 generations; more toes on the rear than on the front feet were white. Very few of the heterozygotes were progeny tested to confirm their genotypes. The backcross parents were chosen from the 29 double heterozygotes of $\underline{Mi}^{wh}$ and Sp since they were easier to classify; the coronal area was either restricted or completely white. Fourteen of the double heterozygotes showed primary breaks ventrally. Many Splotch homozygotes were viable to term in both the NAW coisogenic strain and the marker strain from which it was obtained. According to previous reports all homozygotes died by the 14th day of gestation (Auerbach, 1954).

NAW-+wa-2+To (N6)

Although "Tortoiseshell" is not a piebald spotting mutant, it is included in this study because it is variegated in coat texture and color. Dr. Willard F. Hollander backcrossed To for eight generations to the NAW strain. He gave some of the backcrossed mice to the writer to use in linkage studies of Ames dwarf (df). In the To segregates of the NAW strain, the mutant regions cover far less than one-half of the coat

surface. In heterogeneous stocks the amount of mutant area varies from very little to nearly all of the coat. The animals having the most mutant tissue appear to be weak and frequently die at a young age. The ratios of 22 To:18 non-To females in the NAW strain and 48 To:65 non-To females in the heterogeneous stocks evidently reflect the inviability of some females which have large amounts of mutant tissue. Crosses of NAW-+To (N4-N5) females to C57BL/6 males produced 15 To and 21 non-To females. Less than half of the area of the coats showed the mutant phenotype in the To hybrids.

NAW-+wa-2+Va (N9) and crosses

In addition to the pigment area restriction given in Table 1, the feet were always white in "varitint-waddler" homozygotes. After generation N5 there were no dorsal pigment breaks among the pigment areas on the body. The white of the coronal area sometimes extended posteriorly between the aural centers; nasal and temporal areas were often restricted. A Va homozygote and two heterozygotes from the NAW-a^e-+Va (N7) stock are shown in Fig. 35. The homozygotes described by Cloudman and Bunker (1945) had pigment only at the bases of the ears and tail, but in the NAW coisogenic strains homozygotes had pigment in all the primary areas except the coronal. It was apparent from the phenotypes that the heterozygotes and homozygotes in the NAW strain did not overlap in pigmentation patterns.

Most of the Va homozygotes and many heterozygotes produced no offspring. Only one of the homozygous females which did have litters was able to raise her young. This homozygote from NAW-a^e+Va (N4)

produced 7 heterozygotes (2 mosaic, 5 non-mosaic) from a NAW-a^e (N6) male and 6 heterozygotes (5 mosaic, 1 non-mosaic) from an ENA male. The fact that the offspring were all heterozygotes confirms the classification of the homozygotes by phenotype. One homozygous male of heterogeneous background sired a litter of 7 heterozygotes from a non-Va female and thus demonstrated that homozygous males as well as females can be fertile.

Double heterozygotes obtained in crosses of Va and A^y were variegated and became nearly white with age. The non-Va spots were, of course, yellow rather than non-agouti. A Va/+ male from NAW-Va (N8) bred to C57BL/6 females produced 6 hybrid Va/+ progeny. Three of the hybrids were not variegated when classified at three weeks of age but did exhibit small non-Va spots as adults.

NAW-+wa-2+W (N15) and Z-c^+-+W (N15)

Heterozygotes of "dominant spotting" generally exhibited white on all of their feet. After generation N9 the only other region in which white appeared was on the tail (Table 1). The amount of white on the tails of the NAW heterozygotes varied from the tail tip only to approximately the distal one-third of the tail. In the Z strain, heterozygotes frequently overlapped wild-type; none showed more white than the distal one eighth of the tail. During the backcrossing to the Z strain, many animals whose tails were completely pigmented were suspected of being heterozygous because of the number of toes which were white. When at least three toes on one rear foot and one or more on the other were white, the animal was classified as a heterozygote. After the N6

generation, 4 males and 17 females were classified as heterozygotes by the criterion of white toes. Two of the males and 12 of the females were progeny tested in backcrosses to the Z strain; all proved to be heterozygous.

NAW-+wa-2+W^a (N14), Z-c^+-+W^a (N17) and MWA-+W^a-a^eA^y (N2)

In the NAW strain heterozygotes of "Ames dominant spotting" all had white tail tips, white on all feet, coronal restriction and occasionally ventral primary breaks in pigmentation (Table 1). The same description held in the Z strain except that some of the animals did not exhibit coronal restriction.

One female heterozygote in the Z strain had a fully pigmented tail. From one-sixth to one-half of the distal part of the tail was white in her 17 contemporaries of the N11 generation; in fact there was no tendency toward normal overlap in any generation. When the anomalous female was backcrossed to a Z male, she produced 7 heterozygotes among the non-albino offspring; from one-fourth to one-half of the distal part of the tail was white on all 7 heterozygous offspring.

The roan pattern of the $\underline{W}^a$ heterozygotes in the MWA-$\underline{W}^a\underline{A}^y$ (N2) strain was eliminated when $\underline{A}^y$ was included in the genotype. The interaction was similar to that reported for $\underline{W}$ and $\underline{A}^y$ (Dunn et al., 1937). Two offspring of the first backcross of $\underline{A}^y$ are shown in Fig. 40, $\underline{a}^e\underline{a}^e$ $\underline{W}^a$/+ on the left and $\underline{a}^e\underline{A}^y$ $\underline{W}^a$/+ on the right.

Viability of W and W^a Homozygotes

Prenatal mortality in matings of heterozygous males with heterozygous sisters and non-mutant sisters is given for the NAW and Z coisogenic strains in Table 2. In both strains there was a lower percentage of viable W^a than of W homozygotes.

Table 2. Effect of dominant spotting on prenatal mortality in matings of heterozygous males with heterozygous females and non-spotted sisters of the females

Strain	Heterozygous dams				Non-spotted dams		
	Dead embryos		Live embryos		Dead embryos		Live
	Early	Late	Normal	Anemic	Early	Late	embryos
NAW-W (N6-N13)	25	23	356	104	29	12	625
	4.9%	4.5%	70.1%	20.5%	4.4%	1.8%	93.8%
Z-W (N8-N13)	38	16	192	60	27	4	271
	12.4%	5.2%	62.7%	19.6%	8.9%	1.3%	89.7%
NAW-W^a (N6-N11)	16	6	104	22	16	7	281
	10.8%	4.0%	70.3%	14.9%	5.3%	2.3%	92.4%
Z-W^a (N8-N14)	56	10	145	17	29	8	354
	24.6%	4.4%	63.6%	7.5%	7.4%	2.1%	90.5%

A comparison of the postnatal viability of W and W^a homozygotes is shown in Table 3. Two W homozygous females, which were viable to maturity, were produced in the Z-W (N11) matings. The parents of the viable homozygotes were two separate pairs from one litter. Each of the four parents was backcrossed again to the Z strain; the heterozygous offspring within each of the four resulting families were bred together. Although from 6 to 13 homozygotes were classified in each family, not one

reached maturity. In Table 3 excessive numbers appear in the Z-W row since the homozygotes from each of the families are also included. The two viable females and the female which lived to 7-9 days were among the first 10 homozygotes obtained in the Z-W strain. Thus, the W homozygotes were far more viable than the W^a homozygotes when compared in the Z coisogenic strains. Although there was little difference in the range of

Table 3. Longevity of anemic mice from matings of heterozygous dominant spotting males with heterozygous females and non-spotted sisters of the females

Strain	Age when anemics were last observed alive								Non-anemic	
	1-3 days		4-6 days		7-9 days		2 mo.			
	M	F	M	F	M	F	M	F	M	F
NAW-W (N11)	10	6	2						84	91
Z-W (N11-N13)	36	29	7	7	1	1		2	265	293
NAW-W^a (N11)	3	5	2	1					8	15
Z-W^a (N11-N14)	4	7							40	58

longevity between W and W^a, a higher percentage of W^a homozygotes lived to the age of classification in the NAW coisogenic strains.

Development of Pigment Areas in MSC, MSL and MWA-+W^a Stocks

Some of the stocks were particularly useful for demonstrating the autonomous development of pigment areas. Plate 3 shows the development of the masal area. The MSL stock was selected for complete pigmentation

Plate 3. Delimitation of the nasal pigment areas

Fig. 23. MSL stock (aa ss), absence of both nasal pigment areas

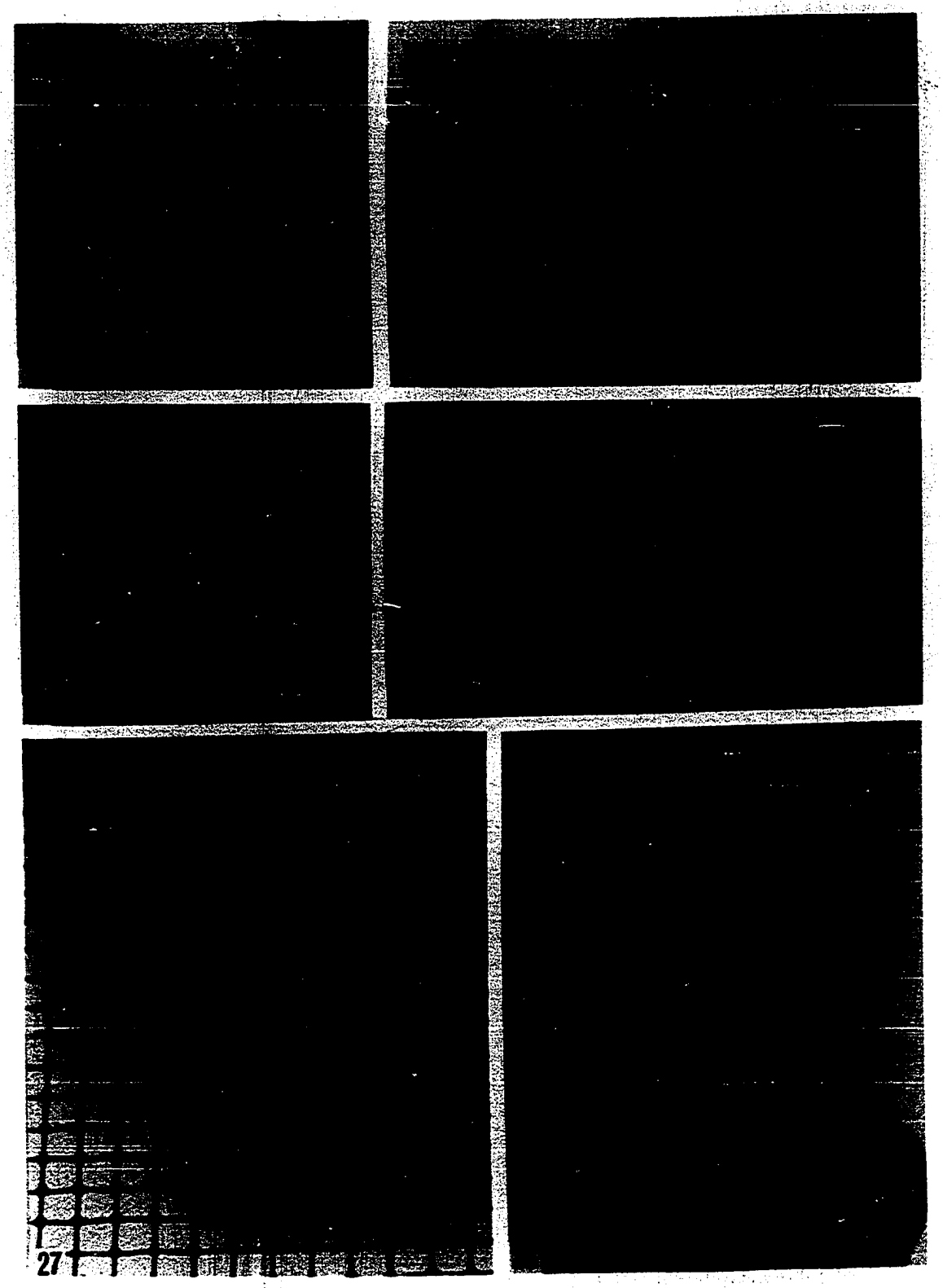
Fig. 24. Left: MSL stock (aa ss), absence of one nasal pigment area. Right: MSC stock (aa ss), complete development of one nasal pigment area on otherwise unpigmented face

Fig. 25. MWA-+W^a stock, development of a non-W^a nasal pigment area on a W^a/+ mouse

Fig. 26. MSL stock (aa ss Mi^{wh}/+), bilateral and unilateral development of nasal pigment areas against an unpigmented background

Figs. 27 and 28. MWA-+W^a stock, dorsal and ventral views of a W^a/+ mosaic in which the coronal and both temporal and both aural pigment areas are non-W^a

Only the nasal pigment areas remain unaltered on the face.


27

of the facial pigment areas (mouse on right, Fig. 20), but occasionally one or both nasal pigment areas did not develop (mouse on left in Fig. 24 or mouse in Fig. 23). In Fig. 26 one or both nasal areas are well developed, but one temporal area is the only other pigment area represented. In contrast, the MSC stock was selected for the absence of facial pigment areas (Fig. 15), but occasionally one of the nasal areas developed (mouse on right in Fig. 24). A genetic change in an early melanoblast presumably gave rise to a nasal pigment area populated with non-$\underline{W}^a$ melanocytes in the $\underline{W}^a$ heterozygote shown in Fig. 25. Conversely, only the nasal areas remained unchanged on the face of the $\underline{W}^a/+$ mosaic in Fig. 27. Thus the nasal pigment area was defined in four different ways: (1) absence of the nasal pigment area on an otherwise pigmented face, (2) presence of the nasal area on an otherwise unpigmented face, (3) presence of a non-$\underline{W}^a$ nasal pigment area on a $\underline{W}^a$ heterozygote and (4) presence of $\underline{W}^a/+$ nasal areas on an otherwise non-$\underline{W}^a$ face.

Mosaicism of $\underline{W}^a$ heterozygotes resulted in patterns similar to those found for $\underline{Mi}^{wh}$ heterozygotes (Plate 1). Examples of the autonomous development of each of the pigment areas except the coronal were found among the $\underline{W}^a$ mosaics. Some of them are shown in Plate 4 and Fig. 25. The coronal area is rarely pigmented in the $\underline{W}^a$ heterozygotes of the MWA-+$\underline{W}^a$ stock, but was pigmented in the mosaic shown in Fig. 27. Apparently non-$\underline{W}^a$ pigment can develop in the coronal area if the surrounding areas (temporal and aural) are also non-$\underline{W}^a$.

Plate 4. Development of pigment areas in $\underline{W}^a/+$ mosaic mice from MWA-+$\underline{W}^a$ stock

Fig. 29. Temporal area

Fig. 30. Aural area

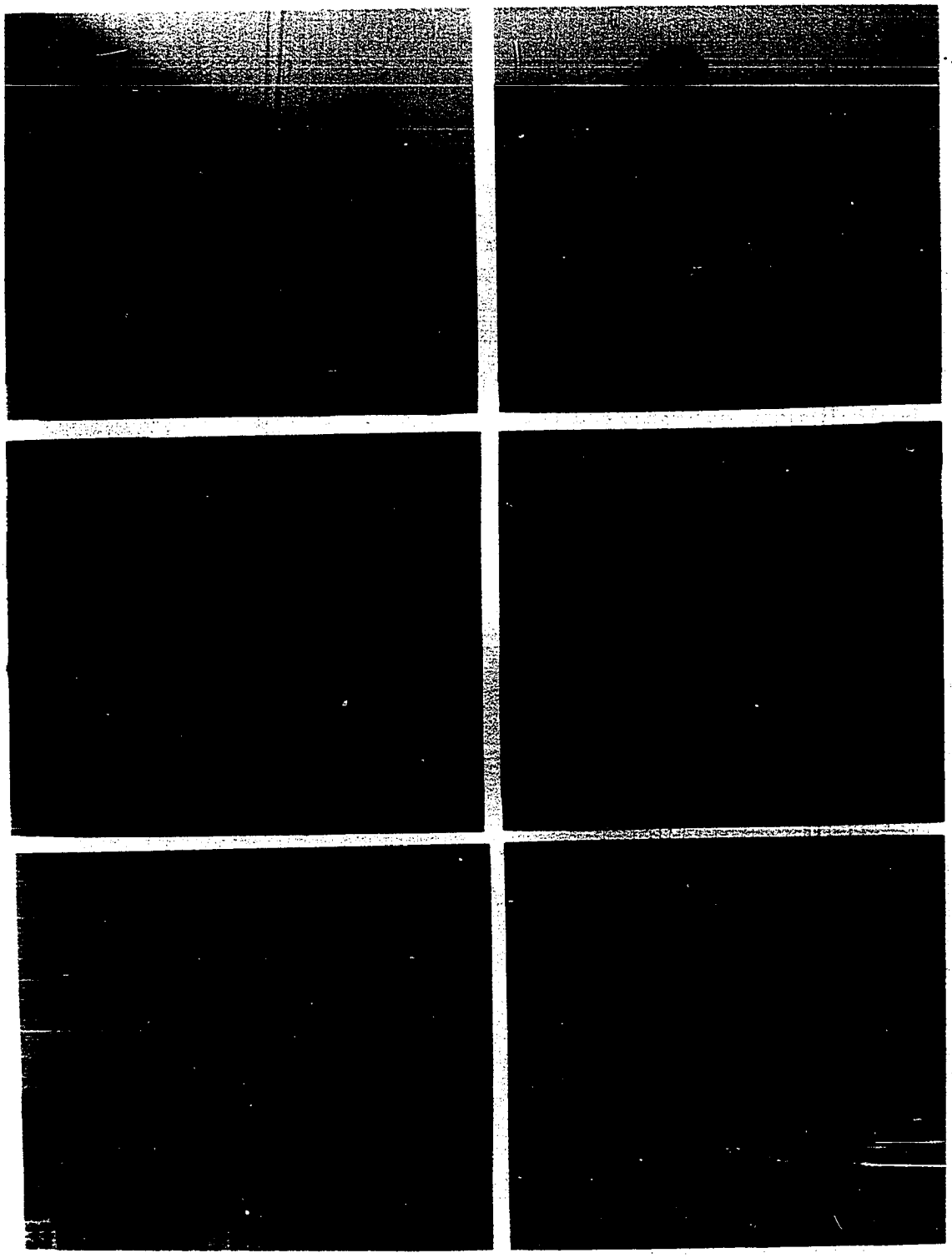
Fig. 31. Sacral area

Fig. 32. Costal area

Fig. 33. Left aural and incomplete right aural areas

Fig. 34. Dorsal view of right aural and left costal areas

Notice that the non-$\underline{W}^a$ area tends to spread over the dorsal midline.



Experiments on the Frequencies of Mosaicism

Comparisons among Mi^{wh}, Mi^{wh} bt, Mi^{wh} s, W^a and Va stocks

The frequencies of $\underline{Mi}^{wh}$/+ mosaics in the various crosses and stocks are given in Table 4. In general, the genotypes which showed the greatest restriction of the pigment areas had the lowest frequency of mosaics. The heterogeneity chi-square for the groups of non-Mos stocks was significant ($P < .005$). The groups were arranged according to genotype (bt/+, btbt, s/+ or ss in combination with $\underline{Mi}^{wh}$/+) and each compared with the standard group ($\underline{Mi}^{wh}$/+, non-bt, non-s). The ss group was the only one in which the frequency of mosaics was significantly lower ($P < .0005$) than the standard. Only the bt/+ group showed a higher frequency of mosaics; however, the difference was very slight.

The frequency of mosaicism in Va heterozygotes was significantly higher ($P < .02$) in crosses than in the NAW-+Va strain (Table 5). It was obvious that the non-Va areas were larger in the heterogeneous crosses (Figs. 36 and 37) than in the inbred strain (Fig. 35). The non-Va pigmentation of the Va/+ mouse in Fig. 37 covered a larger area than in any other Va mosaic observed. Although ninety percent of the heterozygotes were listed as mosaics, many of those which were classified as not mosaic according to their juvenile coats showed small non-Va patches in their adult coats. It appeared that the dark gray color of the juvenile heterozygotes (Fig. 35) was unstable. Most of the coat became off-white in the adult, but small patches of non-Va pigment indicated that changes occurred in both directions. Occasionally the change to off-white was clearly visible in the juvenile coat. The off-white areas did

Table 4. Frequencies of $Mi^{wh}/+$ mosaics in piebald spotted and non-piebald spotted matings

Genotype		Strain or stock	$Mi^{wh}/+$ offspring				Freq. of Mos	X^2
			Mosaic		Non-mosaic			
Sire	Dam		M	F	M	F		
$Mi^{wh}/+$	+	NAW-+Mi^{wh}	4	4	67	68	.056	
$Mi^{wh}/+$	+	ENA	4	4	141	119	.030	
+	$Mi^{wh}/+$	NAW-+Mi^{wh}	3	1	56	52	.036	
+	$Mi^{wh}/+$	ENA	1	6	140	126	.026	
$Mi^{wh}/+$	$Mi^{wh}/+$	Crosses	11	13	199	224	.054	
$Mi^{wh}Mi^{wh}$	+	Crosses	15	14	186	209	.068	
+	$Mi^{wh}Mi^{wh}$	Crosses	10	10	204	184	.049	
			48	52	993	983	.048	9.61
$Mi^{wh}Mi^{wh}$	btbt	Crosses	8	15	204	189	.055	
btbt	$Mi^{wh}Mi^{wh}$	Crosses	14	14	215	232	.059	
$Mi^{wh}Mi^{wh}$ btbt	+	Crosses	10	7	207	209	.039	
+	$Mi^{wh}Mi^{wh}$ btbt	Crosses	12	9	197	173	.054	
			44	45	823	803	.052	2.01
$Mi^{wh}/+$ btbt	btbt	MBT	0	1	38	46	.012	
btbt	$Mi^{wh}/+$ btbt	MBT	2	3	58	58	.041	
$Mi^{wh}/+$ btbt	$Mi^{wh}/+$ btbt	MBT	7	5	175	197	.031	
			9	9	271	301	.031	1.50
$Mi^{wh}Mi^{wh}$	ss	Crosses	14	9	218	205	.052	
ss	$Mi^{wh}Mi^{wh}$	Crosses	12	9	218	229	.045	
$Mi^{wh}Mi^{wh}$ ss	+	Crosses	7	10	272	271	.030	
+	$Mi^{wh}Mi^{wh}$ ss	Crosses	9	8	216	204	.039	
			42	36	924	909	.041	3.13
$Mi^{wh}/+$ ss	ss	MSC	1	3	45	55	.038	
$Mi^{wh}/+$ ss	ss	MSL	1	1	78	61	.014	
ss	$Mi^{wh}/+$ ss	MSC	1	1	52	59	.018	
ss	$Mi^{wh}/+$ ss	MSL	0	0	76	83	.000	
$Mi^{wh}/+$ ss	$Mi^{wh}/+$ ss	Crosses	2	2	229	253	.008	
			5	7	480	511	.012	8.98

Table 4. (Continued)

Genotype Sire	Genotype Dam	Strain or stock	Mi^{wh}/+ offspring Mosaic M	Mosaic F	Non-mosaic M	Non-mosaic F	Freq. of Mos	X^2
Mi^{wh}/+ mos	+	Mos	9	12	154	135	.068	
Mi^{wh}/+ mos	+	bt^+ Mos	10	10	215	194	.047	
Mi^{wh}/+ mos	+	s^+ Mos	3	7	96	99	.049	
+	Mi^{wh}/+ mos	Mos	7	8	95	106	.070	
+	Mi^{wh}/+ mos	bt^+ Mos	9	5	144	118	.051	
+	Mi^{wh}/+ mos	s^+ Mos	6	8	100	130	.057	
Mi^{wh}/+ mos	Mi^{wh}/+ mos	Mos	14	7	162	145	.064	
			58	57	966	927	.057	2.89
Mi^{wh}/+ bt/+ mos	+	bt Mos	35	36	538	582	.060	
+	Mi^{wh}/+ bt/+ mos	bt Mos	31	27	391	377	.070	
			66	63	929	959	.064	.92
Mi^{wh}/+ s/+ mos	+	s Mos	7	8	179	172	.041	
+	Mi^{wh}/+ s/+ mos	s Mos	8	11	190	180	.049	
			15	19	369	352	.045	.27
Heterogeneity		Total	287	288	5755	5745	.048	73.62[a]
Heterogeneity	Groups of non-Mos stocks		148	149	3491	3507	.041	31.29[a]
bt/+ vs non-bt, non-s group			92	97	1816	1786	.050	.28
btbt vs non-bt, non-s group			57	61	1264	1284	.044	3.39
s/+ vs non-bt, non-s group			90	88	1917	1892	.045	1.26
ss vs non-bt, non-s group			53	59	1473	1494	.036	25.26[a]
Heterogeneity	Groups of Mos stocks		139	139	2264	2238	.058	3.64
Mi^{wh}/+	+	Total	74	86	1551	1532	.049	
+	Mi^{wh}/+	Total	68	70	1302	1289	.051	
			142	156	2853	2821	.050	.04

[a] $P < .0005$.

Table 4. (Continued)

Genotype Sire	Genotype Dam	Strain or stock	Mi^{wh}/+ offspring Mosaic M	Mosaic F	Non-mosaic M	Non-mosaic F	Freq. of Mos	X^2
$Mi^{wh}Mi^{wh}$	+	Total	54	55	1087	1083	.048	
+	$Mi^{wh}Mi^{wh}$	Total	57	50	1050	1022	.049	
			111	105	2137	2105	.048	.04
Mos, non-bt, non-s group vs non-Mos, non-bt, non-s group			106	109	1959	1910	.053	1.70
Males vs		Total	287		5755		.048	
Females		Total		288		5745	.048	.00

Table 5. Frequencies of Va/+ mosaics

Genotype Sire	Genotype Dam	Strain or stock	Va/+ offspring Mosaic M	Mosaic F	Non-mosaic M	Non-mosaic F	Freq. of Mos	X^2
Va/+	+	NAW→+Va	64	60	11	8	.867	
+	Va/+	NAW→+Va	19	28	2	4	.887	
			83	88	13	12	.872	.13
Va/+	+	Crosses	51	43	4	1	.949	
+	Va/+	Crosses	12	10	0	0	1.000	
			63	53	4	1	.959	1.16
Heterogeneity		Total	146	141	17	13	.905	7.20
NAW→+Va vs Crosses		Total	146	141	17	13	.905	6.49[a]
Va/+	+	Total	115	103	15	9	.901	
+	Va/+	Total	31	38	2	4	.920	
			146	141	17	13	.905	.24
Males vs		Total	146		17		.896	
Females		Total		141		13	.916	
			146	141	17	13	.905	.36

[a] $P < .02$.

Plate 5. Va mice

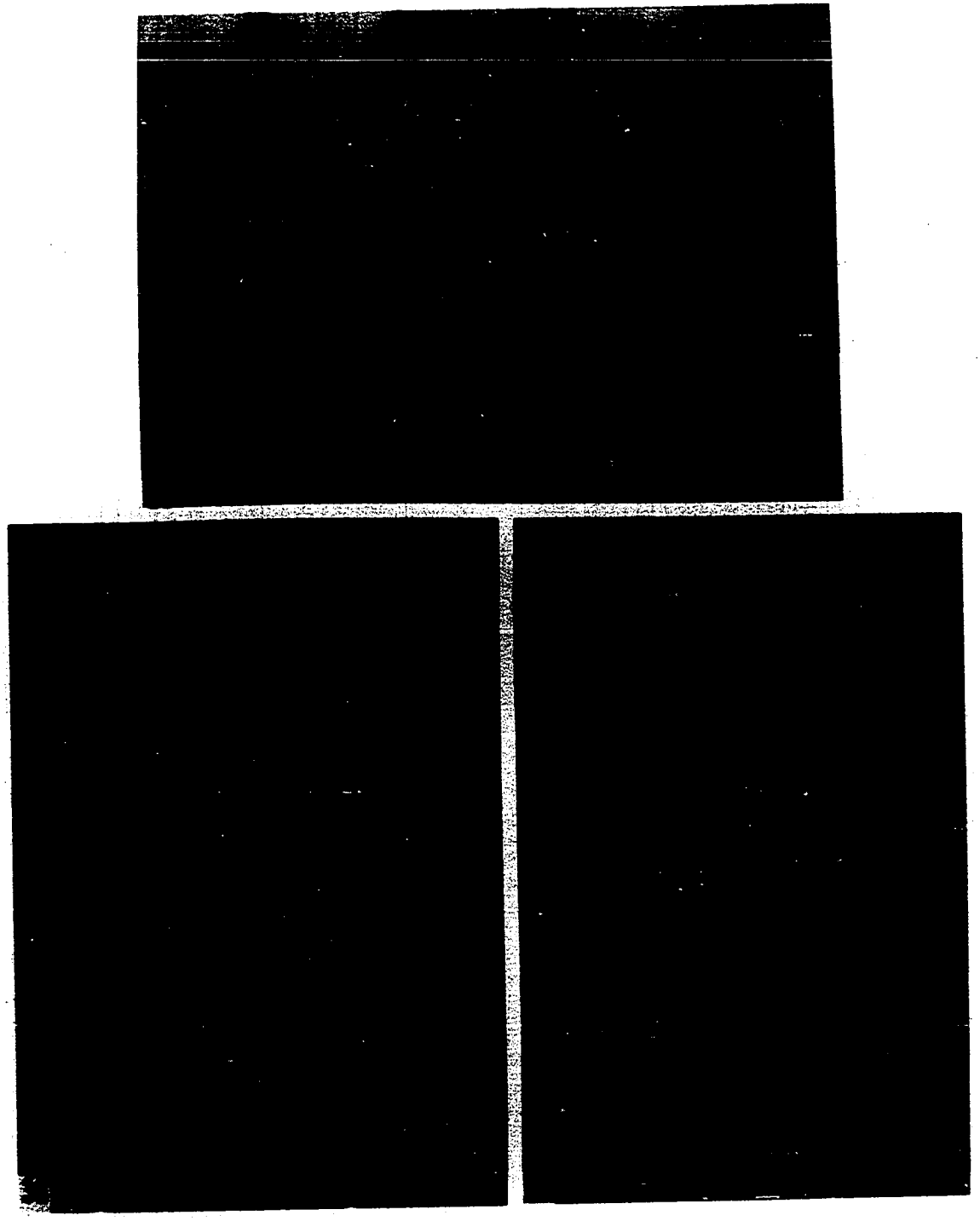
Fig. 35. NAW-a^e--+Va (N7) littermates: Va/+ mice at each end, VaVa mouse left center, non-Va mouse right center

Notice the two levels of gray pigment on the homozygote.

Fig. 36. Va/+ mice from crosses: wild-type aural and costal pigment areas

These mice carry no mutant allele at the non-agouti locus.

Fig. 37. Va/+ mouse from crosses: the non-Va area is the most extensive that was observed in this study



not correspond to the primary breaks in pigmentation, as shown in Fig. 7, but were usually within pigment areas. The off-white areas continued to appear on the dorsums of Va heterozygotes in the NAW-+Va strain even after generation N5 when there were no longer any dorsal primary pigment area breaks occurring. Homozygotes became nearly white as old adults but showed evidence of mosaicism in the juvenile coat in that two shades of gray could be distinguished (Fig. 35).

The data in Tables 4, 5 and 6 for Mi^{wh}, Va and W^{a} respectively show that none of the following factors had a significant effect on the frequency of mosaicism at any of the three loci: (1) selection for mosaicism, (2) transmission of mutant gene through sire versus dam and (3) sex of the heterozygote.

The relative numbers of genetic changes which took place within the single pigment areas of each genotype under study were tabulated in Table 7. All of the single area mosaics that were sketched, including those from non-pedigreed stocks and those classified when over 30 days of age, are accounted for. Mosaics in which more than one pigment area was non-mutant were excluded. In all, there were 893 single area and 56 multiple area $Mi^{wh}/+$ mosaics and 178 single area and 13 multiple area $W^{a}/+$ mosaics.

Note that no genetic changes took place in the lumbar areas of the MBT stock. In comparison with the non-bt, non-s mice, the difference is significant ($X^2 = 9.97$, $P < .003$).

More genetic changes of single areas occurred on the right than on the left. Of the $Mi^{wh}/+$ mosaics which could be definitely classified as right or left, 320 were on the right and 260 on the left ($X^2=6.21$, $P<.02$).

Table 6. Frequencies of $\underline{W}^a$/+ mosaics

Genotype		Strain or stock	W^a/+ offspring				Freq. of Mos	X^2
			Mosaic		Non-mosaic			
Sire	Dam		M	F	M	F		
$\underline{W}^a$/+	+	MWA	12	15	124	131	.096	
+	$\underline{W}^a$/+	MWA	16	11	139	129	.092	
$\underline{W}^a$/+	$\underline{W}^a$/+	MWA	8	12	96	124	.083	
			36	38	359	384	.091	.25
$\underline{W}^a$/+ mos	+	MWA Mos	24	17	242	249	.077	
+	$\underline{W}^a$/+ mos	MWA Mos	14	10	175	190	.062	
			38	27	417	439	.071	.81
Heterogeneity		Total	74	65	776	823	.080	3.35
Mosaic matings vs non-mosaic matings		Total	74	65	776	823	.080	2.35
Males vs		Total	74		776		.087	
Females		Total		65		823	.073	
			74	65	776	823	.080	1.13
$\underline{W}^a$/+	+	Total	36	32	366	380	.084	
+	$\underline{W}^a$/+	Total	30	21	314	319	.075	
			66	53	680	699	.079	.41

Table 7. Relative numbers of genetic changes taking place within single pigment areas

Stock	Genotype	Nas	Temp	Cor	Aur	Cos	Lum	Sac	Cd
Crosses	$\underline{Mi}^{wh}$/+	3 1%	20 8%	10 4$	28 12%	41 17%	43 18%	41 17%	57 23%
Crosses	$\underline{Mi}^{wh}$/+ $\underline{bt}^{+}$	5 3%	9 5%	10 6%	24 13%	22 12%	30 17%	45 25%	35 19%
Crosses	$\underline{Mi}^{wh}$/+ $\underline{s}^{+}$	3 3%	4 4%	2 2%	18 17%	11 11%	25 24%	25 24%	16 15%
Crosses	$\underline{Mi}^{wh}$/+ bt/+	4 2%	15 9%	11 7%	27 17%	21 13%	21 13%	43 27%	19 12%
Crosses X-ray	$\underline{Mi}^{wh}$/+ bt/+	2 5%	3 8%	1 3%	5 14%	11 30%	3 8%	7 19%	5 14%
MBT	$\underline{Mi}^{wh}$/+ btbt	1 2%	7 15%	7 15%	8 17%	7 15%		12 25%	6 12%
Crosses	$\underline{Mi}^{wh}$/+ s/+	4 4%	5 5%	3 3%	4 4%	22 22%	16 16%	23 23%	21 21%
Crosses	$\underline{Mi}^{wh}$/+ ss				2 17%		4 33%	6 50%	
MSC	$\underline{Mi}^{wh}$/+ ss					3 38%	3 38%	2 25%	
MSL	$\underline{Mi}^{wh}$/+ ss					1 50%		1 50%	
MWA	$\underline{W}^{a}$/+	17 10%	20 11%	2 1%	32 18%	41 23%	29 16%	26 15%	11 6%

The difference was not significant in the $\underline{W}^a/+$ mosaics, but there were 87 altered areas on the right and 76 on the left.

Only the mosaics which showed non-mutant areas were included in Table 7. In addition there were 6 $\underline{W}^a/+$ mosaics in which the altered area was some shade of gray between the roan color of the heterozygote and the black color of the non-$\underline{W}^a$ mouse. There were 37 $\underline{Mi}^{wh}/+$ mosaics in which the altered area was lighter than the background color and 23 in which the area was darker but still not as dark as non-$\underline{Mi}^{wh}$. Two $\underline{Mi}^{wh}/+$ mosaics showed both light and non-$\underline{Mi}^{wh}$ areas, one of which is shown in Fig. 38. The light areas tended to be more yellow than gray in color as shown also in Fig. 39.

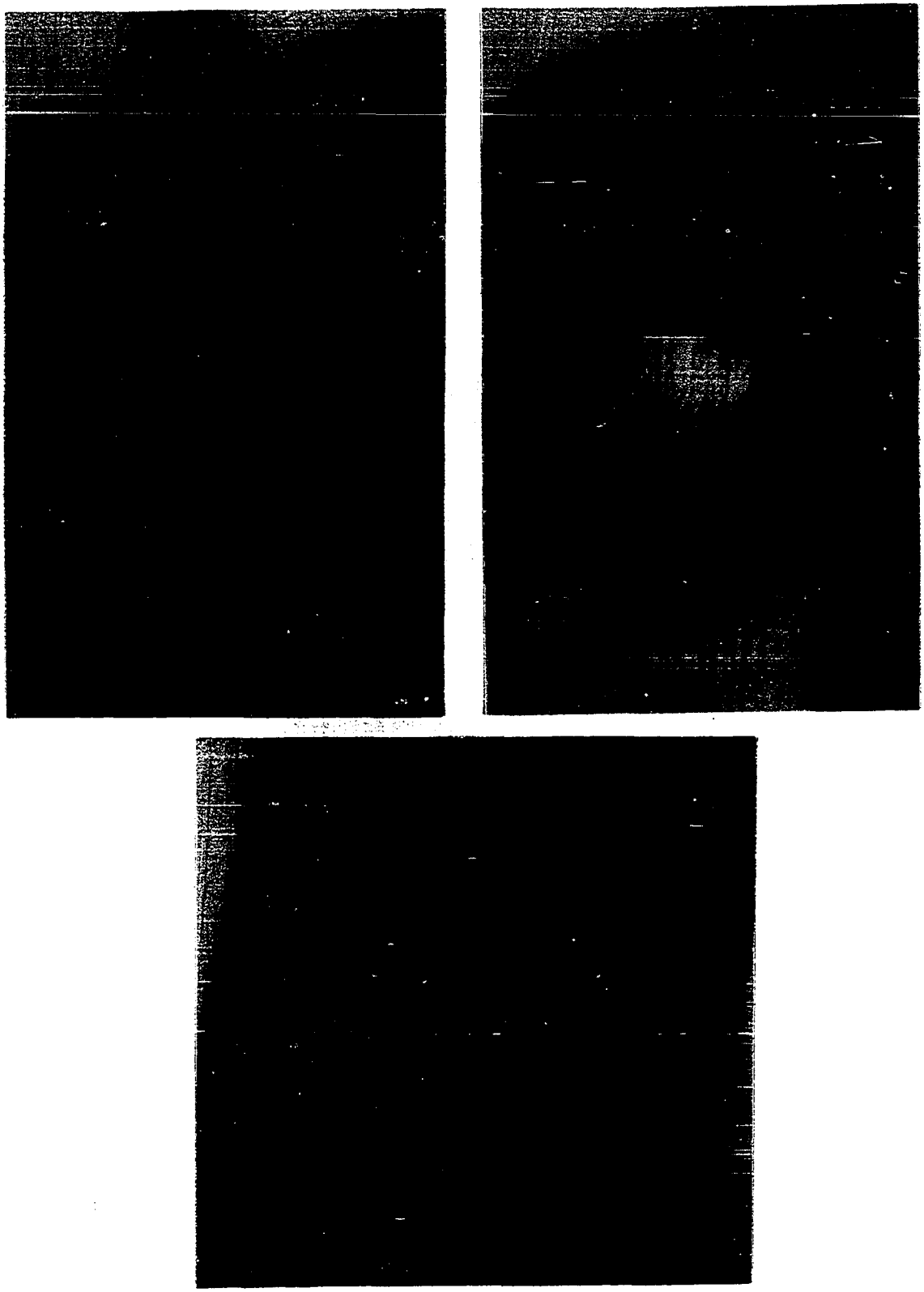
The light and dark $\underline{Mi}^{wh}/+$ mosaics were tested for recessive color mutations by breeding them to sibs, progeny or suspected mutants. Of the 5 dark mosaics which were tested to some degree, one was heterozygous for pink-eyed dilution (p) and one for brown (b). Of the 19 tested light mosaics, 7 were heterozygous for p, b, leaden (ln) or albino (c). Two of the dark mosaics were tested with enough sibs and progeny that the probability was less than .006 and .04 that they carried undetected recessives. The probability was less than .0008 and .05 for two light mosaics and less than .04 for the light mosaic from the MBT stock shown in Fig. 39. The probability was actually much less for the MBT mosaic since no color mutant other than $\underline{Mi}^{wh}$ had segregated in the MBT stock during the year prior to the occurrence of the mosaic or during the two years that have followed. At least six pairs of the MBT stock have been producing young continuously. The probability was less than .04 that the

Fig. 38. Left: Mosaic showing a coronal area of lighter shade than the $\underline{Mi}^{wh}/+$ background and also a non-$\underline{Mi}^{wh}$ patch in the right aural area

Fig. 39. Right: MBT stock ($\underline{a}^{e}\underline{a}^{e}$ $\underline{btbt}$ $\underline{Mi}^{wh}/+$) mosaic in which the left costal area is a lighter shade than the background phenotype

Fig. 40. MWA-+$\underline{W}^{a}$-$\underline{a}^{e}\underline{A}^{y}$ (N2) littermates

Notice that the roan effect in the $\underline{W}^{a}$ heterozygote on the left was eliminated when yellow ($\underline{A}^{y}$) was combined with $\underline{W}^{a}/+$ in the mouse on the right.



mosaic in Fig. 38 carried any recessive color mutations.

Frequencies in Mi^{wh}/+ bt/+ mice X-rayed in utero

The data presented in Table 8 show that the frequencies of mosaics are not the same for each stage of irradiation, the probability of the heterogeniety chi-square being less than .025. Treatment at days 2.5 and 10.5 showed a significant increase ($P < .02$ and $< .003$ respectively) above the control, but overall frequency was not increased.

Table 8. Frequencies of mosaics from matings of $Mi^{wh}Mi^{wh}$ males with X-rayed btbt females

Days pregnant when X-rayed	Mosaics M	Mosaics F	Non-mosaics M	Non-mosaics F	Freq. of Mos	X^2	P
.5	0	0	1	5	.000		
1.5	0	0	6	6	.000		
2.5	1	2	3	6	.250		
3.5	1	0	6	4	.091		
4.5	4	5	34	45	.102		
5.5	2	0	14	11	.074		
6.5	2	1	14	11	.107		
7.5	3	1	19	25	.083		
8.5	3	2	35	38	.064		
9.5	1	1	28	26	.036		
10.5	6	6	23	13	.250		
11.5	0	0	5	3	.000		
Heterogeneity	23	18	188	193	.097	22.49	< .025
Control	7	13	103	98	.090		
X-rayed vs control	30	31	291	291	.095	.06	
10.5 vs control	13	19	126	111	.119	9.57	< .003
2.5 vs control	8	15	106	104	.099	5.78	< .02

The frequency of mice showing dorsal restriction of the lumbar areas was significantly higher ($P < .0005$) in the X-rayed mice than in the controls (Table 9). The males showed greater restriction than the females ($P < .05$). The lumbar areas were restricted ventrally in nearly all of the mice; hence there were no differences among groups.

Table 9. Frequencies of dorsal restriction of lumbar pigment areas in offspring of $\underline{Mi}^{wh}\underline{Mi}^{wh}$ males and X-rayed $\underline{btbt}$ females

Days pregnant when X-rayed	Restricted M	Restricted F	Non-restricted M	Non-restricted F	Freq. rest.	X^2	P
.5	1	5	0	0	1.000		
1.5	5	6	1	0	.917		
2.5	4	5	0	3	.750		
3.5	6	3	1	1	.818		
4.5	33	44	5	6	.875		
5.5	11	5	5	6	.593		
6.5	13	9	3	3	.786		
7.5	23	22	1	2	.938		
8.5	37	36	1	4	.936		
9.5	24	25	5	2	.875		
10.5	25	13	4	6	.792		
11.5	5	3	0	0	1.000		
Heterogeneity	187	176	26	33	.860	29.56	< .0009
Control	86	75	24	36	.729		
X-rayed vs control	273	251	50	69	.815	16.68	< .0005
.5 - 6.5	73	77	15	19	.815		
7.5 - 11.5	114	99	11	14	.895		
	187	176	26	33	.860	5.48	< .025
.5 - 6.5 vs control	159	152	39	55	.768	4.24	< .05
7.5 - 11.5 vs control	200	174	35	50	.815	21.04	< .0005
Males	273		50		.845		
Females		251		69	.784		
	273	251	50	69	.815	3.94	< .05

Tests for twin spots

Although there were 13 $\underline{Mi}^{wh}$ mosaics among the 272 offspring from the cross of $\underline{Mi}^{wh}\underline{Mi}^{wh}$ X ss wa-1wa-1, there were no wa-1 mosaics. There were 5 $\underline{Mi}^{wh}$ but no wa-1 mosaics among the 158 offspring from the crosses of $\underline{Mi}^{wh}$ wa-1/$\underline{Mi}^{wh}$ wa-1 X +.

Tests for germinal mosaicism

The segregation ratios for heterozygotes of $\underline{Mi}^{wh}$, Va and $\underline{W}^{a}$ are given in Tables 10, 11 and 12 respectively. The data are broken down into mating types and stocks or strains within types, each line of the table being one category. Although there were numerous matings in each category, only the number of those which produced at least 20 offspring is listed in next to the last column. A chi-square, including the correction for continuity, was calculated for each mating, but is not given in the table. The total chi-square for the matings which produced at least 20 offspring is given in the last column. As expected, there were a few statistically significant chi-squares among the matings; 15 among a total of 290 $\underline{Mi}^{wh}$ matings, 2 among 24 Va matings and 3 among 88 $\underline{W}^{a}$ matings. However, since the heterogeneity chi-square was not significant at the P=.05 level for any category of matings, it is assumed that the few matings which produced abnormal ratios were simply chance errors. Unfortunately a second set of at least 50 offspring could not be obtained from any one of the anomalous matings. In most cases the ratio was nearly 1:1 in the few offspring which were obtained in the second set. The mosaic which produced the most deviant ratio was a $\underline{Mi}^{wh}$/+ female from the $\underline{s}^{+}$Mos stock; she produced 34 $\underline{Mi}^{wh}$/+ : 16 + in the first set

Table 10. Segregation ratios of $\underline{Mi}^{wh}/+$ mice

Genotype Sire	Genotype Dam	Strain or stock	Offspring $\underline{Mi}^{wh}/+$	Offspring +	X^2	Pooled X^2	Matings[a] No.	Matings[a] Total X^2
$\underline{Mi}^{wh}/+$	+	NAW→$\underline{Mi}^{wh}$	228	207	1.01		9	7.46
		ENA	344	361	.41		14	13.95
		MBT	136	169	3.57		7	6.00
		MSC	155	140	.76		6	4.67
		MSL	217	209	.15		10	6.04
$\underline{Mi}^{wh}/+$ mos	+	Mos	377	383	.05		10	8.58
		$\underline{bt}^{+}$ Mos	506	486	.40		18	7.11
		$\underline{s}^{+}$ Mos	253	264	.23		8	7.12
		$\underline{bt}/+$ Mos	1406	1375	.35		48	36.57
		$\underline{s}/+$ Mos	470	445	.68		17	16.67
+	$\underline{Mi}^{wh}/+$	NAW→$\underline{Mi}^{wh}$	152	166	.62		7	2.80
		ENA	338	262	9.63	(P<.004)[b]	15	15.57
		MBT	166	171	.07		7	6.26
		MSC	149	155	.12		9	9.15
		MSL	242	259	.58		10	6.27
+	$\underline{Mi}^{wh}/+$ mos	Mos	254	231	1.09		12	5.28
		$\underline{bt}^{+}$ Mos	294	274	.70		13	15.40
		$\underline{s}^{+}$ Mos	301	252	4.34	(P<.04)[b]	11	18.64
		$\underline{bt}/+$ Mos	959	929	.48		42	29.77
		$\underline{s}/+$ Mos	429	425	.02		17	16.87
			7376	7163	25.26	3.12	290	240.17
Mosaic parents		Total	5249	5064	8.34	3.32		
Non-mosaic parents		Total	2127	2099	16.92	.19		
$\underline{Mi}^{wh}/+$ sires		Total	4092	4039	7.62	.35		
$\underline{Mi}^{wh}/+$ dams		Total	3284	3124	17.64	4.00 (P<.05)[b]		

[a]Only matings which produced at least 20 offspring were included. X^2 is the sum of the chi-squares for the individual matings.

[b]Probability for the X^2 to the immediate left.

Table 11. Segregation ratios of Va/+ mice

Genotype Sire	Genotype Dam	Stock	Offspring Va/+	Offspring +	X^2	Pooled X^2	Matings[a] No.	Matings[a] Total X^2
Va/+	+	Crosses	371	428	4.07[b]		19	12.67
+	Va/+	Crosses	123	114	.34		5	1.57
			494	542	4.41	2.22	24	14.24

[a]Only matings which produced at least 20 offspring were included.

[b]$P < .05$.

Table 12. Segregation ratios of W^a/+ mice

Genotype Sire	Genotype Dam	Stock	Offspring W^a/+	Offspring +	X^2	Pooled X^2	Matings[a] No.	Matings[a] Total X^2
W^a/+	+	MWA	255	277	.91		9	10.66
W^a/+	+	Crosses	262	303	2.98		13	11.46
W^a/+ mos	+	MWA Mos	542	538	.01		28	18.01
+	W^a/+	MWA	268	280	.26		11	3.36
+	W^a/+	Crosses	146	150	.05		8	.08
+	W^a/+ mos	MWA Mos	382	392	.13		19	21.56
			1855	1940	4.34	1.90	88	65.13
Mosaic parents		Total	924	930	.14	.02		
Non-mosaic parents		Total	931	1010	4.20	3.22		
W^a/+ sires		Total	1059	1118	3.90	1.60		
W^a/+ dams		Total	796	822	.45	.42		

[a]Only matings which produced at least 20 offspring were included.

(X^2=5.78, P<.02) and 22 $\underline{Mi}^{wh}$/+ : 12 + in the second set (X^2=2.38, P<.14).

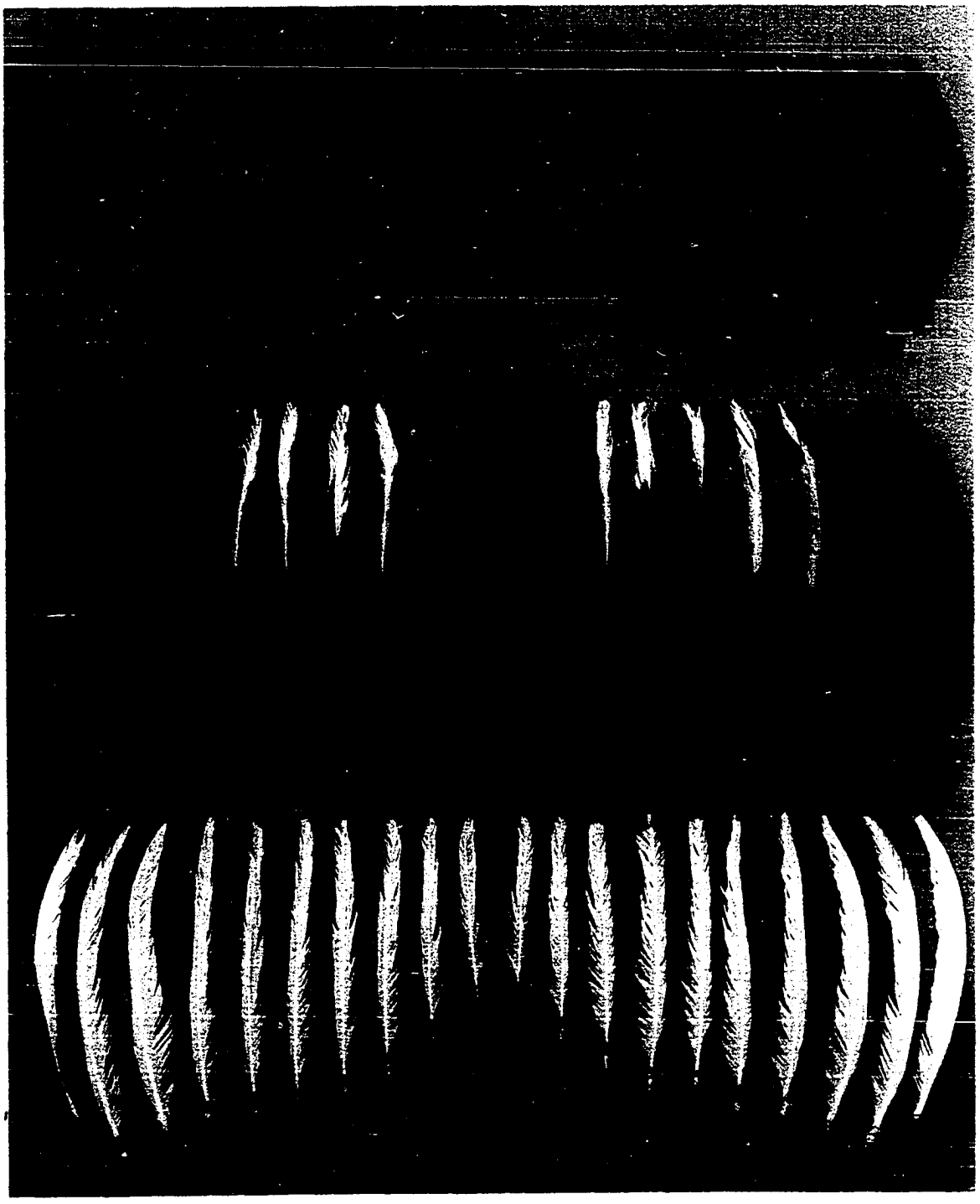
Although none of the total ratios differed significantly from 1:1, the ratios obtained from $\underline{Va}$/+ males and $\underline{Mi}^{wh}$/+ females did differ. The $\underline{Va}$/+ males produced an excess of non-$\underline{Va}$ offspring; the $\underline{Mi}^{wh}$/+ females an excess of $\underline{Mi}^{wh}$/+ (P<.05 in both cases). A large part of the unbalance in the $\underline{Mi}^{wh}$ ratios was contributed by the ENA and $\underline{s}^{+}$Mos stocks; the latter stock included the mosaic female which produced the most aberrant ratio.

Pigment Spread during Development of the Chicken

Pigment first appeared proximally on the wing and then spread to the tip during the growth of the juvenile and first adult plumage. Although only the remiges were examined specifically, spreading appeared to continue until pigment cells had reached every feather follicle. If spreading took place at all in the unfeathered regions (beak and legs), the process was very slow.

In Fig. 41 spreading of pigment in the primary remiges of the juvenile plumage is compared among the three breeds examined (Black Leghorn, Black Polish Crested and Ancona). The proximal primaries are in the center and the distal ones at each end. The tips of the first six primaries are visible at hatching; pigment had spread to all six on both sides in the Leghorn, to only the first two in the Crested and to none in the Ancona. By the time the seventh primary began to grow pigment had reached that position on the left but not on the right in the Leghorn and Crested. By the time the eighth and all succeeding primaries began to grow in the Leghorn and Crested, pigment had already reached the position

Fig. 41. Comparison of pigment spread in the juvenile primary remiges of Black Leghorn, Black Polish Crested and Ancona (top to bottom), first primaries on the inside and tenth primaries at the ends



each of the follicles. Since the bases of all of the primaries were pigmented in the Leghorn and Crested breeds, it appeared that the pigment invaded the follicle sometime during the growth of the juvenile primary if it were not present when growth was initiated. In the Ancona it appeared that pigment did not reach any of the follicles except the tenth one on the left.

The alternation of pigmented and unpigmented remiges could be very misleading if the time of growth of each feather were not considered. In general the feathers emerged at an earlier age in Ancona chicks than in the White Leghorn, Rhode Island Red or Light Brahma which were examined by Warren and Gordon (1935). The dates of growth were recorded for each feather of the Ancona chicks and the feathers arranged on a time chart. The data and chart for the right wing of an untreated Ancona chick are given in Table 13 and Fig. 42. The proximal feathers begin on the right with the twelfth secondary. The tips of the second through the ninth secondaries were visible at hatching. In the chick of Fig. 42 pigment had spread as far as the sixth secondary at hatching. Apparently it had reached the position of the first secondary when growth began about one week later but had not reached the position of the axial follicle when growth began at about two weeks. Spreading of pigment evidently reached the level of the second primary follicle and all succeeding follicles except the ninth by the time the respective first adult feathers began to grow.

The photographs of feathers (Figs. 21, 22, 41 and 42) illustrate a peculiarity of the Ancona breed which rendered it somewhat less suitable

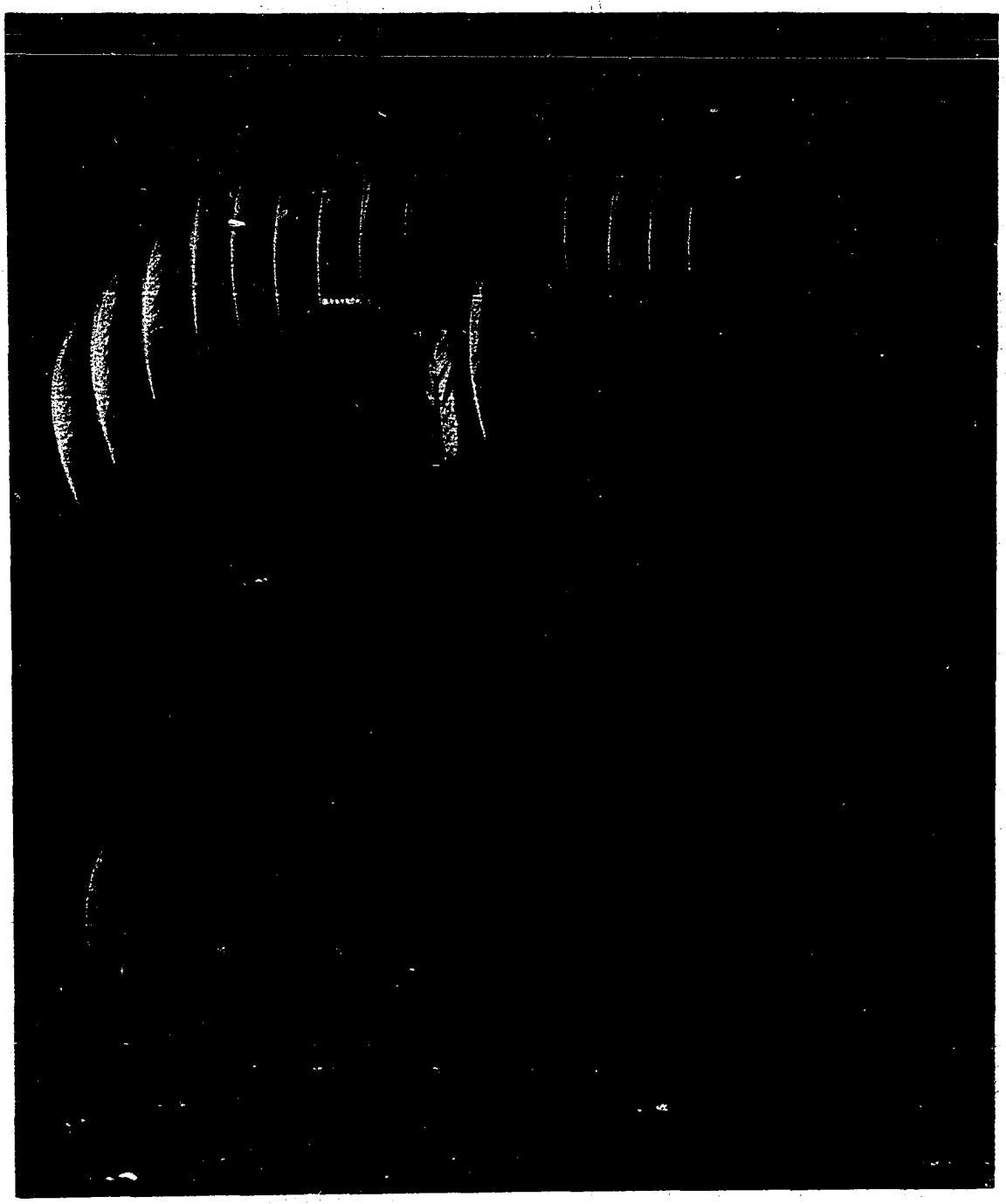
Table 13. Growth stages when pigment first appeared in the remiges of the right wing of an Ancona chick

Feather location	Pigment[a] first observed				Growth periods of feathers[b] Juvenile			Adult		
	Date	S	L	PL	Date	L	Date	Date	L	Date
Primary										
10	11-29	a	1	1/2	7-25	1/2	9-19	11-29	1	
9					7-18	1/2	9-19	11- 1	1	
8	11- 1	a	3	3	7- 7	1/4	9-19	11- 1	3	1- 9
7	10-10	a	p	p	6-28	1/4	8-25	10-10	p	11-29
6	10-10	a	2	2	6-28	1/4	8-17	10-10	2	11-29
5	9-19	a	p	p	6-28	1/4	8- 6	9-19	p	11-29
4	9-19	a	2	2	6-28	1/4	8- 6	9-19	2	11- 1
3	9-19	a	5	5	6-28	1/4	8- 6	9-19	5	10-10
2	8-17	a	p	p	6-28	p	7-25	8-17	p	10-10
1	9-19	a	8	5	6-28	p	7-25	8- 6	1/4	9-19
Axial	7-18	a	3	3	7-18	1	9-19	11-29	3	
Secondary										
1	7- 7	j	1/2	1/4	7- 7	1/2	8-25	10-10	1/4	1- 9
2	8-25	a	1	1	6-28	p	8- 6	8-25	1	10-10
3	8-25	a	p	p	6-28	p	8- 6	8-25	p	10-10
4	9-19	a	4	4	6-28	p	8- 6	9-19	4	11- 1
5	9-19	a	2	2	6-28	p	8- 6	9-19	2	11- 1
6	6-28	j	p	p	6-28	p	8- 6	9-19	1/2	11-29
7	6-28	j	p	p	6-28	p	8-17	10-10	3	11-29
8	6-28	d			6-28	p	8-17	10-10	2	11-29
9	6-28	d			6-28	p	8-17	10-10	1	11-29
10					7- 7	1	8-25	10-10	p	1- 9
11					7- 7	1/2	9-19	11-29	6	1- 9
12					7-25	1	9-19	1- 9	4	

[a]The following record was made when pigment was first observed at a follicle: stage (S) noted as down (d), juvenile (j), or adult (a); length of feather vane (L) in inches or as pinfeather (p); pigmented length of feather vane (PL).

[b]For each juvenile and adult feather, the following information was recorded: date of first observation, length of feather vane (L) in inches or as pinfeather (p) at first observation, date when base of feather became translucent.

Fig. 42. Pigment spread in the remiges of the right wing of an Ancona chicken, juvenile feathers above and first adult feathers below



than the Black Polish Crested for the study of pigment spreading. Notice that no feather of the Ancona chickens was unpigmented in the distal half and pigmented in the basal half as in the Crested chicks. When an Ancona feather was partially pigmented, the pigment extended as a stripe from tip to base and showed only a slight increase in amount at the base. Apparently no pigment cells can enter the follicle during active growth of the feather in the Ancona. The pigmented feathers of the juvenile and first adult plumages of the Ancona differ from those of subsequent adult plumages. In the mature adult, the tips and bases of most feathers are white but the intervening portion is pigmented; in earlier plumages, the entire length of the feather is pigmented.

In most respects the Ancona provided excellent material for the study of pigment spread since restriction could be nearly as extreme as the chick in Fig. 21 which received 200r at the fourth day of incubation. In that chick, right secondaries 9, 10 and 11 were the only juvenile remiges which showed any pigment. Primaries 1 and 2 and secondaries 2 and 3 exhibited little or no pigment in the first adult plumage but were replaced by pigmented feathers in the second adult plumage. All other primary and secondary follicles produced pigmented feathers in the first adult plumage.

When only a few secondaries were the only remiges that were pigmented in the juvenile plumage, the pigmented area on the wing frequently was separated from the pigmented areas on the back. Apparently pigment in the wing spread from a center located near the eighth secondary. If only one secondary were pigmented, it was usually the eighth or ninth,

but in one case the seventh.

The diagram of pigment areas of the chicken (Fig. 43) was the result of studying sketches of 34 Ancona chicks. Since no experimental approach was used to delimit the pigment areas and the number of observed chicks was small, the result is much more subject to error than the diagram for the mouse (Fig. 7).

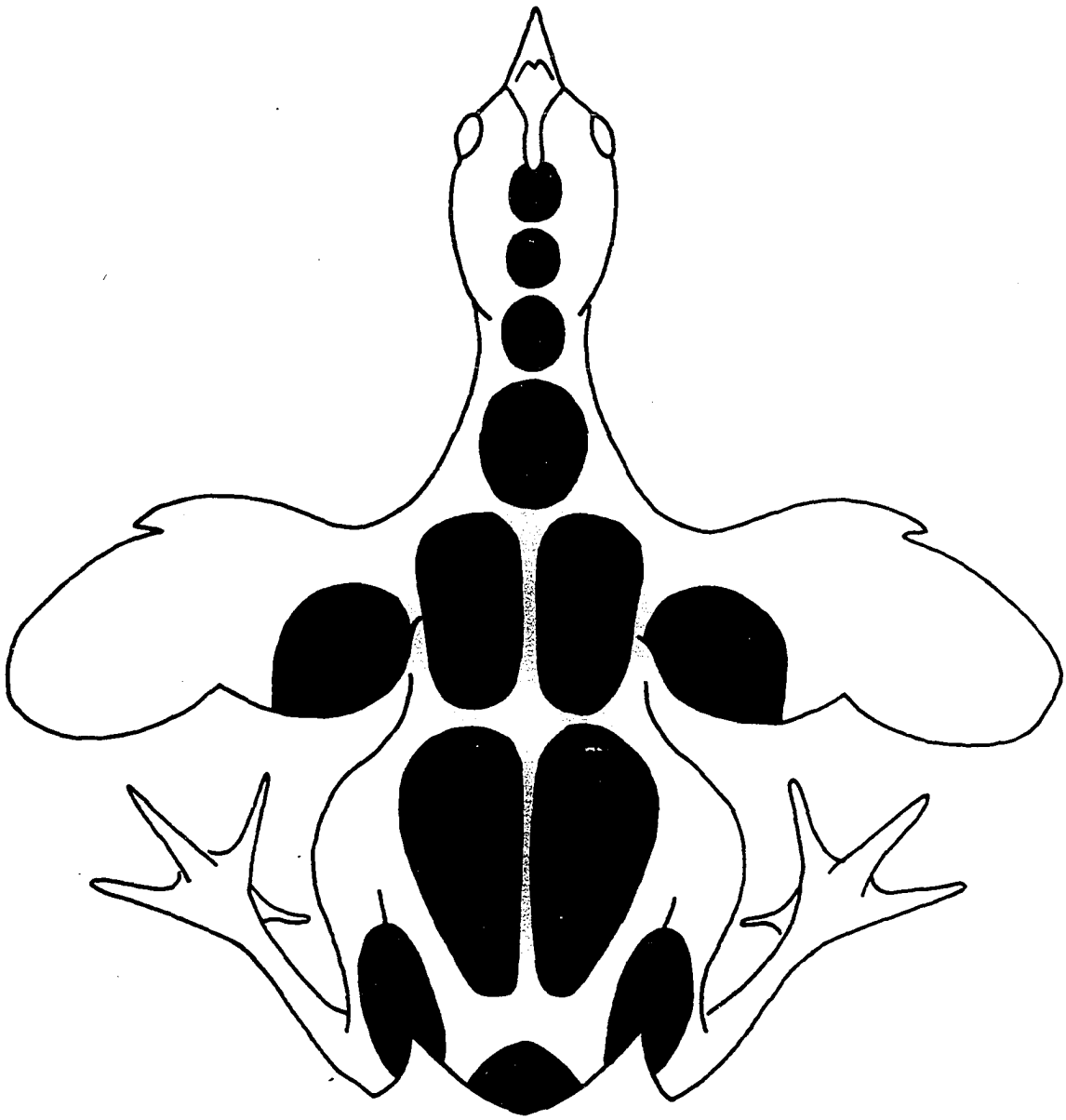


Fig. 43. The most likely arrangement of the pigment centers of the chick as determined from sketches of 34 Ancona chicks

DISCUSSION OF RESULTS

Migration of Pigment Cells during Development

The mouse

It is not known when or where melanoblasts become differentiated from more generalized cells, but it is likely that differentiation takes place within embryonic fields as do many other cell types (Stern 1954a, 1954b, 1955). Transplants of neural tube primordium from the mouse or any area of blastoderm, which is known to form neural tissue, from the chick will differentiate melanocytes. The neural crest is more abundant at some levels than others in the normal chick (Hamilton, 1952). There may be field arrangement in the crest.

The pigmentation of the mouse coat naturally develops from two medial and six bilateral pigment centers. Delimitation of the pigment areas was demonstrated by (1) the absence of a pigment area on a pigmented background, (2) the presence of a pigmented area on an unpigmented background, and (3) the presence of a genetically altered area against the expected phenotype of $\underline{Mi}^{wh}$, $\underline{W}^{a}$ and $\underline{Va}$ heterozygotes. In a very few cases an unaltered area was observed against an altered background, the nasal area of Fig. 27 being an example. Such types were few since only a part of one pigment area was generally altered on any one animal. The absence of an aural, costal or sacral area against a pigmented background could not be demonstrated since no mutant gene is known to specifically restrict pigment spread in those regions in the mouse.

The pigment centers of the mouse shown in Fig. 7 probably hold true

for the wild-type, but it is unlikely that all of the melanocytes proliferated from a center in a wild-type mouse remain within the areas which were defined by observing primary breaks and non-mutant areas in piebald spotted mutants. This conclusion is based on observations of $\underline{Mi}^{wh}$ heterozygotes which in terms of pigment area restriction more closely approach wild-type than the other variegated piebald spotting mutants. Probably all of the non-$\underline{Mi}^{wh}$ melanocytes of the mosaic in Figs. 1 and 2 originated in the right costal center but later mixed with the $\underline{Mi}^{wh}/+$ melanocytes of contiguous areas. It appears that whenever the populations of melanoblasts from adjacent areas meet, there is considerable competition for space. Melanoblasts seem to migrate wherever there is unoccupied territory regardless of their center of origin.

Mixing of non-$\underline{W}^{a}$ with $\underline{W}^{a}/+$ melanoblasts of contiguous pigment areas does not seem to take place in $\underline{W}^{a}/+$ mosaics. Invariably non-$\underline{W}^{a}$ areas have clean cut borders (Plate 4). Apparently the non-$\underline{W}^{a}$ melanoblasts migrate and establish their territory earlier than $\underline{W}^{a}/+$ cells or else the non-$\underline{W}^{a}$ cells can displace the $\underline{W}^{a}/+$ melanoblasts.

Dominance of one type of melanoblast over another in the development of color patterns has been demonstrated in salamanders. Twitty (1949) demonstrated by transplantation experiments that donor melanophores from Amblystoma tigrinum could suppress the activities of the indigenous chromatophores in the slower developing Triturus torsus hosts. Chromatophores of older donors could suppress development of chromatophores of young hosts of the same species. Lehman's (1957) and Lehman and Young's

(1959) observations of Amblystoma mexicanum chromatophores in vitro and in vivo indicated that the migration of melanophores took place before migration of xanthophores. Melanophores migrated first from the neural crest and established clusters in the skin; the clusters became separated as xanthophores passed in between them in their lateroventral migration. As differentiation of the chromatophores proceeded, like types tended to aggregate; the result was a barred or spotted pattern.

The tortoiseshell pattern of guinea pigs described by Wright (1917) is very similar to that of A. mexicanum. The early migration of black melanoblasts followed by the filling in with yellow melanoblasts would explain Wright's observation that the non-yellow pigment tended to be centripital and the yellow pigment centrifugal in the pigmented spots of piebald spotted tortoiseshell guinea pigs. The fact that pigmented areas are larger in black and white than in yellow and white piebald spotted Cocker Spaniels (Little, 1957) indicates that the black melanoblasts are more active than yellow ones in migration.

The generalization canot be made, however, that black pigment cells migrate faster than yellow ones in all species. Lehman and Youngs (1959) observed that the rate of migration was the same for the melanophores and xanthophores of Amblystoma punctatum. The temporary piebald spotting pattern which indicates retarded migration of melanoblasts in E mutant chicks may be a pleiotropic effect of the gene rather than a physiological property of black melanoblasts.

Wright (1942) observed a gradient in the degree of restriction of pigment areas in piebald spotted guinea pigs. The frequency of well

developed pigment areas was highest along the sides of the head; restriction increased posteriorly and anteriorly, the nose being unpigmented the most frequently. In explanation, Wright (1920, p. 324) stated:

> Although the skin of a piebald guinea-pig is divided sharply into areas in which pigment is either produced to the full amount characteristic of the animal, or is wholly absent, it is not to be supposed that the influences which at some critical period in ontogeny determine whether a region is to be colored or white, are so sharply alternative in themselves. It seems more reasonable to suppose that the sums of favorable and unfavorable influences in different parts of the skin could be arranged in a graded series. Doubtless in certain white regions a slight difference in the conditions would have enabled color to develop, while in others, a great change would have been necessary.

The gradient of pigment area restriction is not the same for the piebald spotting types of all species. In the mouse the aural and sacral areas are least likely to be restricted, the coronal and lumbar the most likely, and the intervening and terminal areas intermediate. From casual observation it appears that the pigment areas of the neck or thorax are most likely to be restricted in piebald spotted rabbits, dogs and swine (Dutch rabbits, Boston Terriers and Hampshire swine being examples). It is obvious that the piebald spotting genes which occur in different species affect different pigment areas. Possibly a gene which controls each area could be found in every species provided enough animals were raised or treated with mutagens to obtain the mutants. On the other hand the developmental process might be such that certain pigment areas of each species would always be more likely to receive pigment cells whenever primordial pigment cells were in short supply.

The fact that selection in the MSL stock produced mice in which only

the coronal area was pigmented (Figs. 18 and 20) tends to disprove the possibility that melanoblasts are distributed to certain areas preferentially. It seems more likely that each pigment area represents an embryonic field in the neural crest or neural plate wherein the differentiation of a primordial melanoblast takes place. Stern's (1954a, 1954b, 1955) work on the differentiation of bristles in Drosophila serves as a precedent for such an hypothesis.

The differences in the gradients of pigment area restriction among species probably reflects differences in the frequencies of mutations affecting different regions in each species. For those species in which particular piebald spotting patterns are specified by breed standards, mutations for the restriction of pigment areas in certain regions would be accumulated more than others.

The action of piebald spotting genes apparently determines the gradient. Thus selection in the MSL stock resulted in the coronal area being in the center of a region of minimum rather than maximum restriction. Likewise in the MSC stock the lumbar area is in a region of minimum, rather than maximum restriction. No one gene seems to specifically restrict any one pigment area. Rather, gene action seems to intensify the developmental process which restricts pigment areas in a particular region of the axis, thereby setting up a pair of gradients which restrict the set of pigment areas nearest their origin more than adjacent sets.

It is difficult to determine just what region of the axis is affected by a piebald spotting gene since minor piebald spotting genes appear to be present in every stock. For example, W^a heterozygotes show

restriction of the coronal and caudal areas and ventral restriction of the costal and lumbar areas in most stocks. In the NAW-+$\underline{W}^a$ strain the coronal area was always unpigmented and the tail always restricted, but ventral restriction usually did not occur. Since the coronal area was not restricted in a few individuals of outcrossed stocks, it appeared that the NAW strain must be homozygous for one or more minor piebald spotting genes which insured restriction of the coronal area in $\underline{W}^a$/+ segregates of the NAW-+$\underline{W}^a$ strain. Backcrossing $\underline{s}$ from the MSL stock to NAW demonstrated again that such minor genes did exist in the NAW strain. The coronal area is nearly always pigmented in the MSL stock, which is homozygous for $\underline{s}$, but the coronal area is usually restricted in the $\underline{s}$ segregates of the NAW-+$\underline{s}$ strain. Apparently $\underline{W}^a$ restricts pigment spread to some degree all along the axis and thus reveals the presence of minor genes which tend to restrict pigment spread in the coronal area of the NAW strain.

The lack of a phenotypic effect of $\underline{bt}$ in the NAW-+$\underline{bt}$ strain demonstrates that some piebald spotting genes can be homozygous without showing an effect. The fact that a typical belt pattern was produced in $\underline{Mi}^{wh}$/+ $\underline{btbt}$ mice supports Dunn's (1942) conclusions that (1) the action of one gene may only approach the threshold of phenotypic expression and (2) two or more genes acting together can exceed the threshold and produce a phenotype.

The effect of each of the piebald spotting genes ($\underline{bt}$, headdot, $\underline{s}$, $\underline{Sl}^{gb}$, $\underline{Sp}$, $\underline{W}$ and $\underline{W}^a$) was amplified when it was in combination with $\underline{Mi}^{wh}$/+. Evidently all of these genes affect one developmental process in the same

way. Markert and Silvers's (1956) hypothesis that bt, f, Mi^{wh}, s, Sl, W and W^{v} act to produce piebald spotting in a manner different from Sp seems very unlikely. Rather, migration of melanoblasts appears to be retarded in all of them.

In several other ways the results of the present study support the hypothesis that piebald spotting patterns are the result of retarded migration of melanoblasts (Chase, 1939; Silvers, 1956) rather than the hypothesis that the tissue environment prevents differentiation, migration or survival of melanoblasts in the white regions (Markert and Silvers, 1956). (1) Non-mutant pigment cells spread beyond the usual boundaries of pigmented spots into the white regions of piebald spotted mosaics. (2) Restricting the pigment areas reduces the frequency of mosaicism presumably by reducing the number of melanoblasts which are subject to genetic change. (3) In utero X-ray treatment restricts pigment areas presumably by reducing the population of melanoblasts.

All three variegated mutants (Mi^{wh}, Va and W^{a}) examined in this study are piebald spotted in that the pigment areas are restricted to some degree. When these genes are segregating within inbred strains, the pattern is so consistent that it is possible to predict which regions will be white. The non-mutant areas in mosaic individuals however do not conform to the usual piebald spotting pattern of the mutant. Instead the non-mutant melanocytes completely fill up the pigment area (if the change involves all of the melanocytes of the area), leaving no unpigmented regions. The lumbar area of the $Mi^{wh}/+$ bt/+ mosaic in Fig. 6 provides a good example. In the $W^{a}/+$ mosaic of Fig. 27 all, except the nasal,

pigment areas of the head are non-$\underline{W}^a$. The coronal area, which is always unpigmented in the MWA-+$\underline{W}^a$ stock, is fully pigmented in this mosaic. Since the melanoblasts migrate from the neural crest to the various areas, a genetic change in the crest should in no way affect the tissue of the skin in the coronal area. If the tissue environment of the coronal area of $\underline{W}^a$/+ mice really prevents differentiation, migration, or survival of $\underline{W}^a$/+ melanoblasts, then it should have the same action on non-$\underline{W}^a$ melanoblasts. Apparently the site of gene action is in the neural crest or the melanoblasts themselves.

Combining ss or btbt with $\underline{Mi}^{wh}$/+ not only reduced the size of pigment areas but also reduced the frequency of mosaic mice (Table 4, $P < .0005$ in the case of ss). Although the reduction in frequency of mosaics among the $\underline{Mi}^{wh}$/+ btbt individuals of the MBT stock was not quite significant at the .05 level, the number of genetic changes in the lumbar areas was reduced to zero (Table 7). The lack of mosaicism in the lumbar areas was statistically significant when compared with 17 percent in the $\underline{Mi}^{wh}$/+ stocks ($P < .003$). Apparently combining ss or btbt with $\underline{Mi}^{wh}$/+ reduces the melanoblast population and thus lowers the number of opportunities for genetic changes in melanoblasts. Dunn's (1934) hypothesis that piebald spotting generally increased the frequency of mosaics did not prove to be true for mosaicism of $\underline{Mi}^{wh}$/+ mice.

Restriction of the lumbar pigment areas was significantly greater ($P < .0005$) in $\underline{Mi}^{wh}$/+ bt/+ mice that had been X-rayed in embryonic stages than in the controls. This observation is in agreement with the results obtained by Russell and Major (1957) from a similar experiment. They

offered the explanation that some of the melanoblasts were killed by radiation; the gaps were filled by neighboring ones. Therefore the edge of the pigmented areas did not advance as far as in the controls.

Theoretically, reduction in pigment cell numbers by X-ray damage could only take place after generalized cells had differentiated into melanoblasts. The loss of a cell which is fated to become a melanoblast could be filled by another cell whose potential includes differentiation into a melanoblast. It is less likely that a melanoblast could be replaced if more than one existed in the field at the time of loss. Although Rose's (1957) theory of differentiation was given primarily for the differentiation of tissues, it may be that the differentiation of one generalized cell into a melanoblast will suppress further differentiation into that type of cell in a given embryonic field. Such seems to be the case of bristle differentiation in Drosophila (Stern, 1954a, 1954b). All other melanoblasts that develop in the field would be proliferated from the one primordial melanoblast.

There was significantly greater ($P < .025$) restriction of lumbar pigment areas among those embryos which were X-rayed from day 7.5 to 11.5 compared to earlier treatment. There was very little a priori basis for making the break between days 6.5 and 7.5. Rawles (1947) demonstrated that grafts taken from the primordium of the neural tube at 8 days contained cells capable of differentiating into melanocytes; melanoblasts began migration at 8.5 days. If the assumption could be made that differentiation into melanoblasts takes place between 6.5 and 7.5 days, the data show that irradiation is effective in significantly reducing the

pigment cell population both before and after differentiation, the effect being greater on melanoblasts. The conclusion might be drawn that compensation for death of cells which are destined to become melanoblasts does not take place. It is more likely that radiation slows down cell division at all stages; the melanoblasts are affected more than other cells.

It is curious that the frequency of ventral pigment breaks found by Russell and Major in both irradiated and control C57BL mice was greater in females than in males. The opposite result was found in this study for mutants of genotypes s, Va, W, W^a and Mi^{wh}/+ bt/+ (Tables 1 and 9); restriction of pigment areas was greater in males. Wright's (1942) observation that females showed more pigment area restriction than males in piebald spotted guinea pigs is in agreement with Russell and Major's observations on mice. The mutants were different in all cases, but it is surprising that the opposite relationship held true for all five genotypes in the present study.

The multiple alleles at the mi locus of the mouse may prove to be the best material for determining the action of piebald spotting genes. Four alleles have been described either in this thesis or in the literature (Grüneberg, 1953; Wolfe, 1962). Although all combinations have not been obtained, some of the possible allelic combinations are arranged primarily according to the degree of pigment area restriction in Table 14. All grades of piebald spotting are represented from the slight restriction of pigment areas seen in Mi^{wh}/+ and mi/+, the white coat with light yellow spots in $Mi^{wh}mi^{sp}$ and $Mi^{wh}mi^{bw}$ compounds, the white coat

Table 14. Combinations of the multiple alleles of the mi locus arranged primarily according to the degree of pigment area restriction

Genotype	Eye pigment	Coat pigment area restriction	Coat pigment dilution
mimi	none	completely unpigmented	?
$Mi^{wh}Mi^{wh}$	narrow pigment ring around iris	completely unpigmented	?
$Mi^{wh}mi$	pigment ring around iris	completely unpigmented	?
$mi^{sp}mi$	"some pigmentation"	dorsal flecks of pigmented hairs	?
$mi^{bw}mi^{bw}$	wild-type	occasional animal has dorsal pigmented spot	nearly wild-type
$Mi^{wh}mi^{bw}$	red pupil	all pigment areas restricted	light yellow in juvenile coat fades in adult
$Mi^{wh}mi^{sp}$	"pigmented"	some restriction	light yellow in juvenile coat "sooty" in adult
$Mi^{wh}/+$	red pupil	slight restriction	black pigment diluted to gray
mi/+	red pupil	very slight restriction	nearly wild-type
$mi^{sp}mi^{sp}$	wild-type	wild-type	wild-type

with an occasional nearly wild-type spot in mi^{bw} homozygotes, to the completely unpigmented coats of homozygotes and compounds of Mi^{wh} and mi. Obviously, the unpigmented homozygotes represent an extreme form of the pigment area restriction which is seen in the heterozygotes.

The effect of less pigment area restriction in the compound than in the homozygotes of $\underline{Mi}^{wh}$ and $\underline{mi}^{bw}$ might be considered as overdominance by some investigators. A case of pseudoallelism in which the pigment area restriction in the trans type is more nearly wild-type than in either homozygote may be involved. But until a crossover between $\underline{Mi}^{wh}$ and $\underline{mi}^{bw}$ is obtained, there is no definite evidence for pseudoallelism.

The gross effects of the mi alleles on the reduction of eye pigment as given in Table 14 do not always appear to correspond to the restriction of primary pigment areas. Pigment of the choroid is absent in $\underline{Mi}^{wh}$ heterozygotes (Markert and Silvers, 1956) and presumably in all compounds involving $\underline{Mi}^{wh}$. Grüneberg (1952) described mi heterozygotes as having a red pupilary reflex similar to that of $\underline{Mi}^{wh}/+$; probably melanocytes are also missing in the choroid of mi heterozygotes. Auerbach (1954) stated that pigmentation of the retina begins during the tenth day of gestation. Apparently differentiation and/or proliferation of retinal pigment cells is abnormal in the homozygotes and compounds of $\underline{Mi}^{wh}$ and mi at least by the tenth day since they have been observed to have incomplete retinas (Grüneberg, 1953; Markert and Silvers, 1956). Since no abnormality of the retinal pigmentation has been observed in homozygotes or compounds which exhibit only mild restriction of coat pigment areas, it appears that the gene action responsible for retarding migration from the neural crest takes place after the retinal pigment cells have differentiated. Apparently the complete restriction of coat pigment areas in $\underline{Mi}^{wh}$ and mi homozygotes and compounds is the result of the same gene action at an earlier stage since retinal pigmentation is incomplete.

Genes might be expected to act differently on melanocytes of the coat and retina since the former are derived from the neural crest and the latter arise *in situ*. The graded effects of the *mi* allelic series indicate that the gene action may only appear to be different in the pigment cells of the retina compared with those of the neural crest because of differences in the timing of development. If gene action can reduce proliferation of pigment cells from both the neural crest and retina, reduced proliferation of the pigment layer of the pars iridica could be expected in *mi* mutants. Pierro and Chase (1962) presented evidence that the number of melanocytes in the pars iridica normally increased well into the second month of postnatal life. The same investigators observed that X-irradiation of newborn mice retarded, but did not permanently damage, development of the pars iridica retina. Even though the retina is intact in the *mi* mutants showing mild pigment reduction, $Mi^{wh}/+$ for example (Markert and Silvers, 1956), postnatal stages should be examined for retarded development of the pigment layer of the pars iridica retina.

The light yellow pigmentation seen in some of the compounds (Mi^{wh} mi^{sp}, $Mi^{wh}mi^{bw}$ and $mi^{sp}mi$) was quite surprising particularly when the animals were non-agouti (*a*) or extreme non-agouti (a^{e}). However, Pocock (1911) noted that the extreme dilution of black pigment was yellowish or tan; his examinations of the "white" skins of a wild tiger and a wild cheetah revealed that the yellow background was diluted to white but the black pattern was diluted to tan. Wolfe (1962) noted that the light yellow color of the juvenile coat of $Mi^{wh}mi^{sp}$ compounds became "sooty"

after the first molt. A slight sootiness could also be detected on the $\underline{Mi}^{wh}\underline{mi}^{bw}$ compounds in this study. The sooty effect, rather than mosaicism, may account for the few pigmented hairs found at the base of an ear of one of the compounds.

The chick

The spreading of pigment from the proximal to the distal remiges of Ancona chicks in some cases required the entire six month period during which the juvenile and first adult plumages were growing. Does the slow spreading of pigment really reflect an equally slow migration of melanoblasts out from the neural crest?

Certainly Rawles's (1955b) statement that melanoblasts reach all body regions of the chick embryo by the fourth day of incubation cannot be valid. Reams (1956) observed that pigment cells of the Black Minorca chick migrated into the lining of the abdominal coelom from the ventral dermis around the umbilicus at 7-8 days of incubation and continued spreading in the coelom until the 16th day.

Possibly the spreading of the epidermal population of melanoblasts is independent of the dermal population after a certain stage of embryogenesis. Watterson (1942) observed that melanoblasts crossed the dermoepidermal boundary of the wing bud of the Barred Rock embryo only during the period 80-91 hours incubation. At 16 days incubation there were no melanocytes in the follicles of white down feathers at the tips of the wings even though the dermis was heavily populated. During the growth of the down feather the structure of the dermoepidermal boundary in the follicle was of such a nature that it appeared to prevent

migration of melanoblasts across it. The structure seemed to be more conducive to migration during the growth of the juvenile feathers which were pigmented. Watterson did not consider the possibility that the juvenile feathers at the wing tips became pigmented by melanoblasts proliferated from those that invaded the epidermis at a more proximal location on the wing during the period 80-91 hours.

Reams (1956) observed that melanoblasts from the abdominal coelomic lining of 10 day Black Minorca embryos could invade feather follicles when transplanted to the leg bud of 72 hour White Leghorn hosts but could not invade the follicles when the donors were 12 days or older. During the period 10-12 days the pigment cells of the coelom differentiated from melanoblasts to pigmented melanocytes. The pigmented melanocytes were capable of migrating in the dermis of the host but they could not enter the follicles of the down feathers. It appears that the state of differentiation of the pigment cell is more important than the structure of the dermoepidermal boundary of the down follicle in determining whether or not pigment cells can migrate across.

Fox (1949) tested the epidermis of limb buds of Barred Rock embryos for the presence of melanoblasts by transplanting grafts to the base of the limb buds of White Leghorn embryos. Most of the grafts from the distal third of the limb buds continued to be void of melanoblasts even as late as 117 hours of incubation. In contrast, all of the grafts from the dorsal surface of the basal third contained melanoblasts by the 45 somite stage (about 104 hours); some of the grafts taken from the ventral surface at the same stage remained unpigmented. Development of pigment

in 5 of the 21 grafts from the distal third may have been due to contamination with dermal melanoblasts. A single melanoblast can form a relatively large population of melanocytes in a graft (Reams, 1956).

If migration of melanoblasts over the distance generated from the development of the distal third of the limb bud continued at the same rate as from the neural crest to the basal third, it is questionable that the advance of melanoblasts would reach the rapidly growing apex until some late stage of growth. The distal third of the limb bud in early stages includes much more than the primordia of the distal third of the adult wing. The upper arm and girdle are organized at 72 hours but the more distal parts are formed from the growth of the distal mesoderm and apical ectoderm (Hamilton, 1952). The differential between the advance of melanoblasts and the advance of the apex of the wing bud probably is much greater in the Ancona than in the Barred Rock examined by Fox.

Caldwell and Caldwell (1962) questioned the generally accepted belief that pigment cells complete most of their migration in early embryonic stages. Their report that melanocytes continued to migrate in the fins of some marine fishes well beyond the larval stages was made at the same time that Schaible (1962) reported the slow rate of pigment spread in the Ancona chick. Even though the genetic evidence of this thesis gives strong support for the retarded migration hypothesis for development of piebald spotting patterns in the mouse, transplants or explants of the pigmented and unpigmented regions of piebald spotted embryos of each species should be performed at specific stages to

determine when melanoblasts actually arrive in each area. Such experiments have been done on the Barred Rock chick (Fox, 1949) and the black-eyed white ($\underline{mi}^{bw}$) mouse (Silvers 1961, 1963; Markert, 1960) but the pigment areas are only slightly restricted in the Barred Rock and almost completely restricted in the $\underline{mi}^{bw}$ mouse. Sampling ectoderm from piebald spotting patterns of medium restriction possibly will result in nearly all positive grafts from some regions and all negative grafts from others.

The method of determining the location of pigment areas of the mouse by the use of variegated mutants possibly could be applied to the chick. Introducing blue (Bl) into the Ancona breed by backcrossing should result in variegated chicks exhibiting some entirely non-blue pigment areas. The diagram of pigment areas in the chick (Fig. 43) was determined in this study simply by observation of patterns in the down plumage. No doubt the result is no more accurate than Allen's (1914) description of the mouse pigment areas which he determined in the same way.

Watterson's (1942) comment that most of the melanoblasts entered the epidermis of the wing of the chick primarily at the ulnordial level during the period 80-91 hours incubation gives additional support to the conclusion that a pigment center is located near the eighth secondary remex. The fact that Weston (1962) could trace the migration of melanoblasts labled with tritiated thymidine in the dorsal ectoderm at 70 hours does not preclude invasion of the epidermis of the wing 10-21 hours later. Fox (1949) showed that melanoblasts were present in the ectoderm of the head as early as the 8-9 somite stage (33-38 hours, Hamilton, 1952).

The appearance of the feathers of the Ancona breed indicates that no melanoblasts can enter the follicles when juvenile and first adult feathers are growing. In contrast, it appears that melanoblasts can enter the follicles of the Black Leghorn and Black Polish Crested breeds at any time. Possibly the gene for mottled (mo) in the Ancona breed allows passage of melanoblasts into the follicle only at the start or between periods of feather growth. If the source of melanoblasts in the adult is a reservoir external to the follicle (Cock and Cohen, 1958), the same gene action could account for the unpigmented tips and bases of adult Ancona feathers. A certain period of time would be required for invading melanoblasts to commence production of pigment, particularly if the response were slow in adult tissues. If the number of invading melanoblasts were limited, pigment production might end before growth of the feather was completed as in the hair of the "light" (B^{lt}) mutation of the mouse (Quevedo and Chase, 1958). A greater growth stimulus in the young bird possibly would account for the elaboration of pigment throughout feather growth of the juvenile and first adult plumages.

Most Likely Mechanisms of Mosaicism in Mi^{wh}, W^{a} and Va Mutants

Anomalies of fertilization can be eliminated as likely mechanisms since the genetic changes seem to be limited to relatively late stages in ontogeny. Since there was no evidence of germinal mosaicism, the conclusion can be drawn that genetic change takes place after the separation of the cell lines which give rise to the germ cells and pigment cells. A lack of whole body reversions indicates that genetic change does not take

place before at least one primordial pigment cell has been established.

There were no whole body reversions in a total of 5165 heterozygotes obtained from matings of $\underline{Mi}^{wh}$ with non-$\underline{Mi}^{wh}$ homozygotes. The total value includes those classified when over 30 days of age in addition to the ones in Table 4. Russell and Major (1956) obtained two whole body reversions in a total of 360 progeny produced by homozygous pearl (pe) parents. The frequency of mosaic pe mice (3.7 percent in males and 10.1 percent in females) was nearly the same as the 4.8 percent for $\underline{Mi}^{wh}$ heterozygotes. There should have been ample opportunity for the production of whole body $\underline{Mi}^{wh}$ reversions. Adequate tests for whole body reversions could not be made for Va and $\underline{W}^{a}$; homozygotes of Va are relatively infertile and $\underline{W}^{a}$ homozygotes are lethal. Whole body reversion could have occurred among the wild-type progenies of Va, $\underline{W}^{a}$ and $\underline{Mi}^{wh}$ heterozygotes and their wild-type mates, but it is doubtful. Many of the wild-type segregates were used to test mosaics; none ever produced homozygotes.

If the genetic change does take place prior to the establishment of the pigment cell line, then the change would have to be of a nature that is lethal to the embryo; otherwise whole body reversions would occur. Since the change to the expression of the wild-type allele seems to result in greater activity of the altered melanoblasts in comparison to the heterozygous ones, it is doubtful that the same change would affect other embryonic cells adversely.

If the coat color mosaics of $\underline{W}$ and $\underline{W}^{v}$, reported by Dunn (1937) and Carter (1952) respectively, really were germinal mosaics, it is likely

that the responsible mechanism was different from that of the $\underline{W}^a$ mosaics reported in this study.

The observation that Va homozygotes are variegated leaves no known alternative to somatic mutation (which includes alteration of gene action) as the mechanism of mosaicism. Theoretically Va homozygotes should have shown three intensities of pigmentation corresponding to the effects of VaVa, Va/+ and ++. The areas of the darkest shade were so small that they could not be identified as ++ with any certainty. Some of the homozygotes required as long as one year to become pure white. If VaVa mutates readily to Va/+ in homozygotes, it seems unlikely that the off white color in heterozygotes represents a change from Va/+ to VaVa. It may be that the off white color represents the degeneration of those pigment cells which do not mutate to ++. Microscopic observations of the pigment produced in successive generations of hairs from specific follicles will have to be made in order to determine the nature of the changes.

There were some qualities of the variegation of Va heterozygotes which appeared to be similar in nature to tortoiseshell (To), a sex-linked mutant in the mouse. If the initial expression of Va/+ is assumed to be dark gray and that of To/+ to be wild-type, then it appears that changes in gene activity do not occur until late in ontogeny for both mutants in the coisogenic NAW strains. The wild-type areas which represent a change in the activity of the Va allele and the light colored areas which represent inactivation of the $\underline{To}^+$ allele were generally small in both strains. Late changes in activity were also apparent for both genes in the F_1 Va and To heterozygotes obtained from crosses to the C57BL/6J

strain.

Russell (1963) presented genetic evidence that the inactivated X-chromosome was not uniformly heterochromatic but contained one or more centers from which heterochromatin spread. She estimated the amount of mutant tissue in the variegated patterns determined by autosomal genes that had been translocated to the X-chromosome. The embryonic time of inactivation of translocated genes seemed to depend on the distance from the heterochromatic center on the X-chromosome to the gene involved on the translocated autosomal fragment.

Cattanach (1963) selected for large and small mutant areas in mouse stocks carrying the same rearrangement. Selection for small areas apparently slowed down the spreading of heterochromatin so that inactivation of the rearranged gene took place late in embryogenesis. Reduction in the areas of altered tissue was considered to be an effect of "controlling elements" in maize (McClintock, 1956). It may be that the NAW strain has controlling elements which slowed down the spreading of heterochromatin in the To and Va mutants of the present study.

The hypothesis of Norby et al. (1962) that the piebald spotting gene of cats controls the size of the yellow and non-yellow spots in tortoiseshell cats is untenable. The pigmented spots are large in piebald spotted cats probably as a result of clones of like melanocytes (yellow or non-yellow) tending to stay together. The clones appear to mix up into smaller patches when the populations are contiguous in tortoiseshell cats that are not piebald spotted. The same effect was observed by Wright (1917) for the combination of tortoiseshell and piebald spotting

in the guinea pig.

Variegation by heterochromatization could very well take place in autosomal genes like Va in the mouse since autosomal genes become variegated by that means in Drosophilla virilus (Schneider, 1962). Ohno and Hauschka's (1960) observation that heterochromatic regions in the autosomes of the mouse behave in a fashion similar to the heteropyknotic X-chromosome provides a cytological basis for variegation of autosomal genes in mammals.

Lethality of W^a homozygotes during the first week of age and a lack of pigment in the coat prevented examination of them for mosaicism. Compounds with viable dominant spotting (W^aW^v) would probably survive, but it is doubtful that they would have enough pigment on the body for mosaicism to occur. Silvers (1956) observed that W^v homozygotes showed some pigment only in the skin of the ear tips. Viable dominant spotting was not available for testing in this study.

The compound of $Mi^{wh}mi^{bw}$, however, did consistently show pigmented spots even though one homozygote was always white and the other one rarely showed a small pigmented spot. No definite mosaicism was observed in the 177 compounds. The frequency of mosaic individuals could be expected to be low (about .01) since the restriction of pigment areas of $Mi^{wh}mi^{bw}$ compounds was comparable to Mi^{wh}/+ ss types. Certainly more compounds will have to be observed before the conclusion can be made that $Mi^{wh}mi^{bw}$ compounds are not subject to variegation.

Since it was not possible to demonstrate that homozygotes or compounds of Mi^{wh} or W^a were variegated, a large choice of mechanisms

remain as possibilities. The various anomalies associated with allelic recombination and segregation in mitosis (such as mitotic crossing over, mitotic nondisjunction, mitotic disjunction and mitotic reduction) should be considered since variegation takes place in the heterozygote. Two new cell genotypes would be expected from each of these mechanisms. Homozygous mutant and wild-type tissue should appear in the case of mitotic crossing over or in the case of mitotic disjunction of homologues. Monosomic mutant or wild-type tissue and trisomic tissue carrying one or two doses of the mutant gene should occur in the case of mitotic nondisjunction of sister chromotids in only one homologue. Haploid mutant and wild-type tissues would result from mitotic reduction.

Since no pigment cells develop from the neural crest in $\underline{Mi}^{wh}$ and $\underline{W}^{a}$ homozygotes, it is debatable that homozygous, monosomic or haploid mutant cells could survive to give mutant effects. Stern (1955, 1958) reviewed cases in which cells of lethal types could and could not survive in non-lethal orgaisms or in tissue culture. The fact that the retinal pigment cells of $\underline{W}$ and $\underline{Mi}^{wh}$ homozygotes appear to be normal (Markert and Silvers, 1956) indicates that the pigment cells from the neural crest could survive providing they differentiated and began migration before the genetic change took place.

The fact that some areas do show phenotypes other than wild-type and the expected shade of $\underline{Mi}^{wh}$ and $\underline{w}^{a}$ heterozygotes indicates that the genetic change results in more than one altered phenotype. The pigment of $\underline{Mi}^{wh}\underline{Mi}^{wh}$ melanocytes would probably be yellow since it is yellow in $\underline{Mi}^{wh}\underline{mi}^{bw}$ and $\underline{Mi}^{wh}\underline{mi}^{sp}$ compounds. The shade of yellow would probably be

much lighter than in the compounds since neither $\underline{mi}^{bw}$ nor $\underline{mi}^{sp}$ dilute pigmentation to any extent when homozygous (Table 14). The fact that the yellow areas of the $\underline{Mi}^{wh}/+$ mosaics in Figs. 38 and 39 are darker in shade than the yellow spots of $\underline{Mi}^{wh}\underline{mi}^{bw}$ compounds tends to negate the possibility that the light areas of $\underline{Mi}^{wh}/+$ mosaics are $\underline{Mi}^{wh}\underline{Mi}^{wh}$. It is possible that the light yellow areas represent the $\underline{Mi}^{wh}\underline{Mi}^{wh}/+$ trisomic condition and that the dark gray areas, exhibiting a shade in between $\underline{Mi}^{wh}/+$ and ++, represent the $\underline{Mi}^{wh}/++$ genotype. The dark gray areas of some $\underline{W}^{a}/+$ mosaics may also correspond to a trisomic condition ($\underline{W}^{a}/++$).

Given that variegation could occur in $\underline{Mi}^{wh}\underline{mi}^{bw}$ compounds, interpretation would be nearly as difficult as for $\underline{Mi}^{wh}/+$ heterozygotes. The effect of $\underline{mi}^{bw}$ appears to differ very little from wild-type in regard to pigment dilution. Microscopic and/or biochemical examinations of the altered pigment would have to be made to determine whether it was $\underline{mi}^{bw}$, $\underline{mi}^{bw}/+$, $\underline{mi}^{bw}\underline{mi}^{bw}$, $\underline{mi}^{bw}/++$ or $\underline{mi}^{bw}\underline{mi}^{bw}/+$.

The lack of twin spots tends to disprove the hypothesis that one of the mitotic segregation anomalies is responsible for mosaicism in $\underline{Mi}^{wh}$ and $\underline{W}^{a}$ heterozygotes. The number of $\underline{Mi}^{wh}/+$ mosaics which showed both light and non-$\underline{Mi}^{wh}$ areas was no greater than could be expected by the occurrence of independent events in the same individuals. Because $\underline{Mi}^{wh}/+$ often have yellowish juvenile coats, no attempt was made to record frequency data for the occurrence of mosaics having the light yellow areas, but the most obvious ones were sketched. Nevertheless it appeared that the frequency of light area mosaics was much lower than the frequency of wild-type areas. If the pigment cells of the light colored areas

carried the most abnormal genetic constitutions (such as $\underline{Mi}^{wh}$, $\underline{Mi}^{wh}\underline{Mi}^{wh}$, $\underline{Mi}^{wh}\underline{Mi}^{wh}$/+ or $\underline{Mi}^{wh}$/++), they might have been at a disadvantage while the + or ++ melanoblasts were at an advantage in competing with $\underline{Mi}^{wh}$/+ melanoblasts for space. If pigment cells of dilution mutants generally are at a disadvantage when competing with non-dilution cells, mosaics involving mutant tissue of the dilute type in a non-dilute background probably would occur more frequently in piebald spotted than in non-piebald spotted types. The assumption is that adequate space would be available in the unpigmented regions of piebald spotted animals for the proliferation of dilute pigment cells away from the suppressive effects of normal pigment cells. Dunn (1934, p. 325) suggested "somatic mitosis may more frequently be irregular in animals with white spotting". It may be that the rates of mitoses for dilute and non-dilute pigment cells are more nearly comparable in piebald spotted than in non-piebald spotted genotypes.

Closely situated twin spots could hardly have been expected in the $\underline{Mi}^{wh}$ $\underline{wa\text{-}1}$ double heterozygotes since $\underline{Mi}^{wh}$ affects the pigment cell line and $\underline{wa\text{-}1}$ presumably affects the cells of the hair follicle. A single genetic change would not be likely to affect both cell lines at the same time. The sex-linked mottled ($\underline{Mo}$) alleles of the mouse seem to be the only genes for curly hair that are subject to mosaicism in mammals. Fraser and Short (1958, 1960) noted that all of the hair mosaics reported in sheep involved staple length rather than fiber diameter, density or crimp. Grüneberg (1952) reviewed the skin transplantation experiments of $\underline{wa\text{-}2}$ mice which indicated that gene control may not be cell specific.

Chromosome loss and deletion of small segments involving the $\underline{Mi}^{wh}$ and $\underline{W}^{a}$ loci seem to be unlikely mechanisms. Neither would account for both light and wild-type expression unless the homologue carrying the wild-type allele were also susceptible to loss. Again the light areas would be $\underline{Mi}^{wh}$ in genotype which would be less likely than $\underline{Mi}^{wh}\underline{Mi}^{wh}/+$ from nondisjunction.

Reverse mutation and mutation to another allele at different rates could account for the variegation of $\underline{Mi}^{wh}$ and $\underline{W}^{a}$ heterozygotes. The heterozygous state is a requirement for gene conversion discussed by Green (1960) and for paramutation (Brink, 1960). Gene conversion presumably could only result in changes to wild-type. An intermediate effect would be the result of paramutation. Various allelic states can be attained through the action of suppressing and controlling elements (McClintock, 1956).

If suppressing and controlling elements are involved, the pink-eyed dilution (p) mutant might be useful in experiments on transposition. The locus is subject to mutation in that multiple alleles have occurred spontaneously and as a result of X-ray treatment (Russell and Russell, 1959). Quevedo and Chase (1958) reported that p heterozygotes were distinguishable particularly in combination with dilution mutants. A dilution effect of p in double heterozygotes with $\underline{Mi}^{wh}$ was observed in this study. The dark area in one $\underline{Mi}^{wh}/+$ p/+ mosaic appeared more likely to be a somatic reversion of p than of $\underline{Mi}^{wh}$.

SUMMARY

1. Whiteness in animals can be the result of absence, malfunction or death of pigment cells. In this thesis the term "piebald spotting" was reserved for those types in which no pigment cells have been observed in the white regions; pigmented spots may or may not be present on the unpigmented background.
2. Coat color patterns which are composed of more than one type of pigment (eumelanin and phaeomelanin) or more than one shade of a pigment can be divided into two basic types.
   a. Bicolor: a bilaterally symmetrical pattern that is relatively consistent among individuals having the same gene mutation.
   b. Variegated: parts or the entirety of one or more pigment areas of an individual will be colored differently than the rest; bilateral symmetry is the exception.
3. The pigment of the coat of the mouse spreads out from two medial centers (coronal and caudal) and six bilateral centers (nasal, temporal, aural, costal, lumbar and sacral). In most cases the extent of each area was demonstrated by the development of the same pattern in at least three different ways.
   a. Development of a pigmented spot on an unpigmented background.
   b. Absence of a pigment area in a pigmented background.
   c. Development of a spot from the descendants of a primordial melanoblast which was presumed to have changed to a genotype different from its $\underline{Mi}^{wh}/+$, $\underline{W}^{a}/+$ or $\underline{Va}/+$ background.

4. The development of piebald spotting patterns appeared to be under genetic control as determined by selection for specific patterns and by backcrossing piebald spotting mutants to inbred strains.
   a. The effect of each piebald spotting gene (bt, headdot, s, Sl^{gb}, Sp, W and W^a) was a distinctive and consistent pattern for mutants in an inbred strain.
   b. Presumably all of the piebald spotting genes could complement one another by acting through the same developmental process; pigment area restriction was increased for each mutant when in combination with $Mi^{wh}/+$.
   c. The amount that a pigment area was restricted appeared to depend on an axial gradient which could be altered by mutant genes.
5. Results support the hypothesis that piebald spotting genes retard the proliferation and consequently the migration of melanoblasts from the neural crest to the skin.
   a. The sizes and shapes of the pigmented spots indicate that limited populations of melanocytes are formed by migration in a lateroventral direction from the neural crest region.
   b. The pigment areas expressing only the wild-type allele in Mi^{wh}, W^a and Va heterozygotes often extend beyond the usual borders into the unpigmented regions. Evidently the tissue environment of regions that are usually unpigmented has no property which precludes the invasion or differentiation of melanoblasts.
   c. The frequency of mosaic individuals was significantly less ($P < .0005$) in $Mi^{wh}/+$ ss than in $Mi^{wh}/+$ stocks. In a selected

white belt stock ($\underline{Mi}^{wh}$/+ $\underline{bt}\underline{bt}$) there were no genetic changes in the lumbar areas. The reduction in frequencies of mosaics in types with restricted pigment areas apparently reflects a reduction in the number of melanoblasts which are subject to genetic change.

d. In utero X-irradiation of $\underline{Mi}^{wh}$/+ $\underline{bt}$/+ embryos increased restriction of the lumbar pigment areas. Apparently the death of some melanoblasts slowed down the migration of the entire population.

6. Pigment area restriction was greater in males than in females for all piebald spotting mutants which could be easily classified ($\underline{s}$, $\underline{Va}$, $\underline{W}$, $\underline{W}^{a}$ and $\underline{Mi}^{wh}$/+ $\underline{bt}$/+).

7. Pigment area restriction was only temporary in piebald spotted chicks of the Ancona, Black Leghorn and Black Polish Crested breeds. Pigment spread from the follicle of one remex to another in a proximodistal direction during the growth of the juvenile and first adult plumages.

8. The following account of pigment spread in relation to hair and feather growth is given as an hypothesis to explain differences among species.

   a. Pigment apparently stops spreading only when all of the hairs or feathers of the coat have matured and a period of follicle rest follows.

   b. The developing follicles draw their supplies of pigment cells from the epidermis.

   c. In mice, rabbits and the birds which must fly immediately after

leaving the nest, all follicles of the coat mature at one time and exhaust the epidermal supply of melanoblasts.

d. In the guinea pig, dog and chicken, development of adjacent follicles is staggered during the time that the juvenile and first adult coats are growing. A growth stimulus seems to be present constantly; there is no period of follicle rest. The epidermal supply of melanoblasts is not exhausted at least in the growing animal.

e. The action of piebald spotting genes seems to retard the migration of melanoblasts.

f. The action of a piebald spotting gene in a species, which exhausts its entire epidermal population of melanoblasts during the simultaneous maturation of all its follicles, results in a stable pattern. If the epidermal supply of pigment cells is exhausted before the retarded melanoblasts have had an opportunity to migrate to all regions of the epidermis, then peripheral regions will remain void of pigment cells. Piebald spotted mice seem to be a result of such circumstances.

g. The mature follicle loses its ability to attract epidermal melanoblasts in some of the animals which retain an epidermal supply of pigment cells. If the melanoblasts have not migrated to all regions of the epidermis by the time all of the follicles have matured in piebald spotting mutants, pigment cells continue to spread in the epidermis but do not enter the follicles. A ring of pigmented skin bearing unpigmented hairs develops around

each pigmented spot of fur. The piebald spotting pattern of guinea pigs seems to develop in this manner.

h. The feather follicles of chickens apparently do not lose the ability to attract epidermal melanocytes at least during the growth of the juvenile and first adult plumages. Consequently all follicles finally receive pigment even though migration of melanoblasts may be retarded by the action of piebald spotting genes.

9. Each of 290, 88 and 24 heterozygotes of $\underline{Mi}^{wh}$, $\underline{W}^{a}$ and $\underline{Va}$ respectively produced at least 20 offspring from wild-type mates in test matings for germinal mosaicism. Since the heterogeneity chi-square for each group was not significant, it can be assumed that very few, if any, germinal mosaics occurred.

10. Female $\underline{Mi}^{wh}/+$ mice produced significantly ($P < .05$) more $\underline{Mi}^{wh}/+$ than non-$\underline{Mi}^{wh}$ offspring. Male $\underline{Va}$ heterozygotes sired significantly ($P < .05$) less $\underline{Va}/+$ than non-$\underline{Va}$ offspring. The expected 1:1 ratios were produced by $\underline{Mi}^{wh}/+$ sires, $\underline{Va}/+$ dams and $\underline{W}^{a}/+$ sires and dams.

11. The frequencies of mosaic individuals among heterozygotes of $\underline{Mi}^{wh}$, $\underline{W}^{a}$ and $\underline{Va}$ were .048, .091 and .905 respectively on genetic backgrounds free of any other known piebald spotting genes. There were no whole body reversions in 5165 offspring from matings of $\underline{Mi}^{wh}$ with non-$\underline{Mi}^{wh}$ homozygotes. Many wild-type segregates from $\underline{Mi}^{wh}$, $\underline{W}^{a}$ and $\underline{Va}$ matings were used for breeding but none showed evidence of producing $\underline{Mi}^{wh}$, $\underline{W}^{a}$ or $\underline{Va}$ gametes. Apparently the genetic change does not take place until at least one primordial pigment cell has become

established.

12. In utero X-irradiation of Mi^{wh}/+ bt/+ embryos at different daily stages from .5 to 11.5 days pregnancy did not raise the overall frequency of mosaic individuals significantly above the control frequency of .090. The significant ($P < .003$) increase to a frequency of .250 for treatment at day 10.5 confirms a similar report in the literature.

13. Because variegation was observed in Va homozygotes, somatic mutation (which includes lateration of gene action) seems to be the only possible mechanism of mosaicism. Because of characteristics peculiar to homozygotes of Mi^{wh} and W^{a} and their compounds with other alleles, a similar test could not be made with them. Various mechanisms were discussed but no conclusions drawn to explain the variegation of Mi^{wh} and W^{a} heterozygotes.

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